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Understanding Assembly of AGO2 RISC: the RNAi enzyme: a Dissertation

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UNDERSTANDING ASSEMBLY OF AGO2 RISC: THE RNAi ENZYME

A Dissertation Presented

By

Christian B. Matranga

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The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

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CHAPTER I: Introduction

History of RNA interference and other RNA silencing pathways

In 1990, Richard Jorgensen's lab initiated a study to test if they could create a more vivid color petunia (Napoli et al. 1990). Their plan was to transform plants with the chalcone synthase transgene—the predicted rate limiting factor in the production of purple pigmentation. Much to their surprise, the transgenic plants, as well as their progeny, displayed a great reduction in pigmentation. This loss of endogenous function was termed "cosuppression" and it was thought that sequence-specific repression resulted from over-expression of the homologous transgene sequence. In 1998, Andrew Fire and Craig Mello described a phenomenon in which double stranded RNA (dsRNA) can trigger silencing of cognate sequences when injected into the nematode, *Caenorhabditis elegans* (Fire et al. 1998). This data explained observations seen years earlier by other worm researchers, and suggested that repression of pigmentation in plants was caused by a dsRNA-intermediate (Guo and Kemphues 1995; Napoli et al. 1990). The phenomenon—which soon after was coined RNA interference (RNAi)—was soon discovered to be a post-transcriptional surveillance system in plants and animals to remove foreign nucleic acids.

RNAi is triggered by long dsRNA; however, 21-23nt long RNAs known as small interfering RNAs are the specificity determinants or "guides" which direct regulation of target (Hamilton and Baulcombe 1999; Zamore et al. 2000). Since the breakthrough of RNAi and the findings that small RNAs are the specificity determinants of this pathway,

the field has grown exponentially, both in number of scientists and the overall progress into the mechanism of these silencing pathways. Many new small RNAs have been discovered through basic RNA cloning methods (Lau et al. 2001; Aravin et al. 2001). microRNAs (miRNAs), first discovered in 1993, fine-tune the expression of endogenous RNAs in plants and in animals (Bartel 2004). miRNA biogenesis is similar to that of siRNAs in that they both require Dicer—the enzyme which cuts long dsRNA precursors into short RNA duplexes. Unlike the miRNA pathway, the siRNA pathway is thought to be an ancient mechanism to ward off foreign nucleic acids such as viruses (Waterhouse et al. 1998; Mourrain et al. 2000; Morel et al. 2002; Wang et al. 2006). In 2001, a class of piwi-associated RNAs (piRNAs) was identified in *Drosophila* (Aravin et al. 2001). Through more recent deep-sequencing efforts, piRNAs were recently discovered in the germline of mammals (Girard et al, 2006; Lau et al. 2006; Aravin et al. 2006; Grivna et al. 2006). Similar to siRNAs, piRNAs are thought to regulate the expression of foreign RNAs such as repetitive elements and transposons in *Drosophila* (Aravin et al. 2001), and have been implicated in RNAi and DNA methylation of transposable elements in mice (Aravin et al. 2007).

It was soon discovered that small RNAs were only part of the final equation in RNA silencing. Argonaute proteins are the effectors of silencing pathways, using its small RNA companion to find their target molecules (Hammond et al. 2001; Mourelatos et al. 2002; Vagin et al. 2006). The Argonaute family of proteins is quite complex. They can bind specific structures of small RNA including the 3´ overhangs through their PAZ domain (Yan et al. 2003; Lingel et al. 2003; Ma et al. 2004) and the 5´ phosphate in the

Mid domain (Parker et al. 2005; Ma et al. 2005). Argonaute and Piwi—a subfamily of Argonaute proteins—are enzymes as well; they can cleave the RNA that is bound to its small RNA guide (Liu et al. 2004; Okamura et al. 2004; Lau et al. 2006). Argonautes have other potential functions including those that support RNP assembly (Tahbaz et al. 2004) and enable RNPs to regulate their targets (Kiriakidou et al. 2007).

Small RNA silencing has captivated the scientific world—bringing new genetic tools to model organisms, new explanations for regulatory interactions, new methods to pharmaceutical discovery, and new life to the biotechnology industry where young start-up companies strive to develop siRNA-based drugs. The field has even generated unprecedented discussion in the popular press including the *Wall Street Journal, New York Times*, and *The Economist*. For those of us in the RNA silencing field, the rapid progress in understanding the mechanism and function of small silencing RNAs and the accelerating discovery of new classes of tiny RNA silencers has been very exciting.

Small Silencing RNAs

The Ambros and Ruvkun labs discovered the first microRNA (miRNA) just fourteen years ago (Lee et al. 1993; Wightman et al. 1993). Small interfering RNAs (siRNAs), the small silencing RNAs that mediate RNA interference (RNAi), were identified eight years ago and were subsequently shown in animals to be derived from a longer double-stranded RNA (dsRNA) trigger and to serve as guides for the destruction of complementary mRNA (Fire et al. 1998; Hamilton and Baulcombe 1999). The discovery of siRNAs enabled the application of RNA interference—itself discovered in

plants in 1990 and in animals in 1995—to mammals, much as the identification in 1998 of dsRNA as the trigger of RNAi enabled the widespread use of RNAi in other animals (Napoli et al. 1990; Guo and Kemphues, 1995). Today, tens of thousands of small silencing RNAs have been identified, generating a plethora of small silencing RNA types and sub-types.

Despite their functional and biological diversity, all small silencing RNAs share a common association with a member of the Argonaute family of proteins (Parker and Barford 2006; Seto et al. 2007) (Figure I-1). Through their association with Argonaute proteins, small RNAs take on unique properties that allow them to regulate diverse biological processes. Here, we consider the three dominant classes of animal small silencing RNAs: miRNAs, siRNAs, and piRNAs (Figure I-2 and 3).

miRNAs

miRNAs reside in the genomes of plants, animals, and viruses, but not, we believe, fungi (Lee et al. 1993; Bartel 2004; Pfeffer et al. 2004). miRNAs are transcribed as mRNA-like primary (pri-) miRNAs containing ~70 nucleotide long stem-loop structures (Figure I-2, left) (Han et al. 2004; Lee et al. 2005). Once excised from the pri-miRNA by the nuclear enzyme Drosha, these stem-loops become precursor (pre-) miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm, where they are cleaved again, by the ribonuclease Dicer, to yield ~22 nucleotide long mature miRNAs containing 5´ phosphate and 3´ hydroxy termini (Yi et al. 2003; Lund et al. 2004). A small number of pre-miRNAs also double as introns—"mirtrons"—which are

Figure I-1

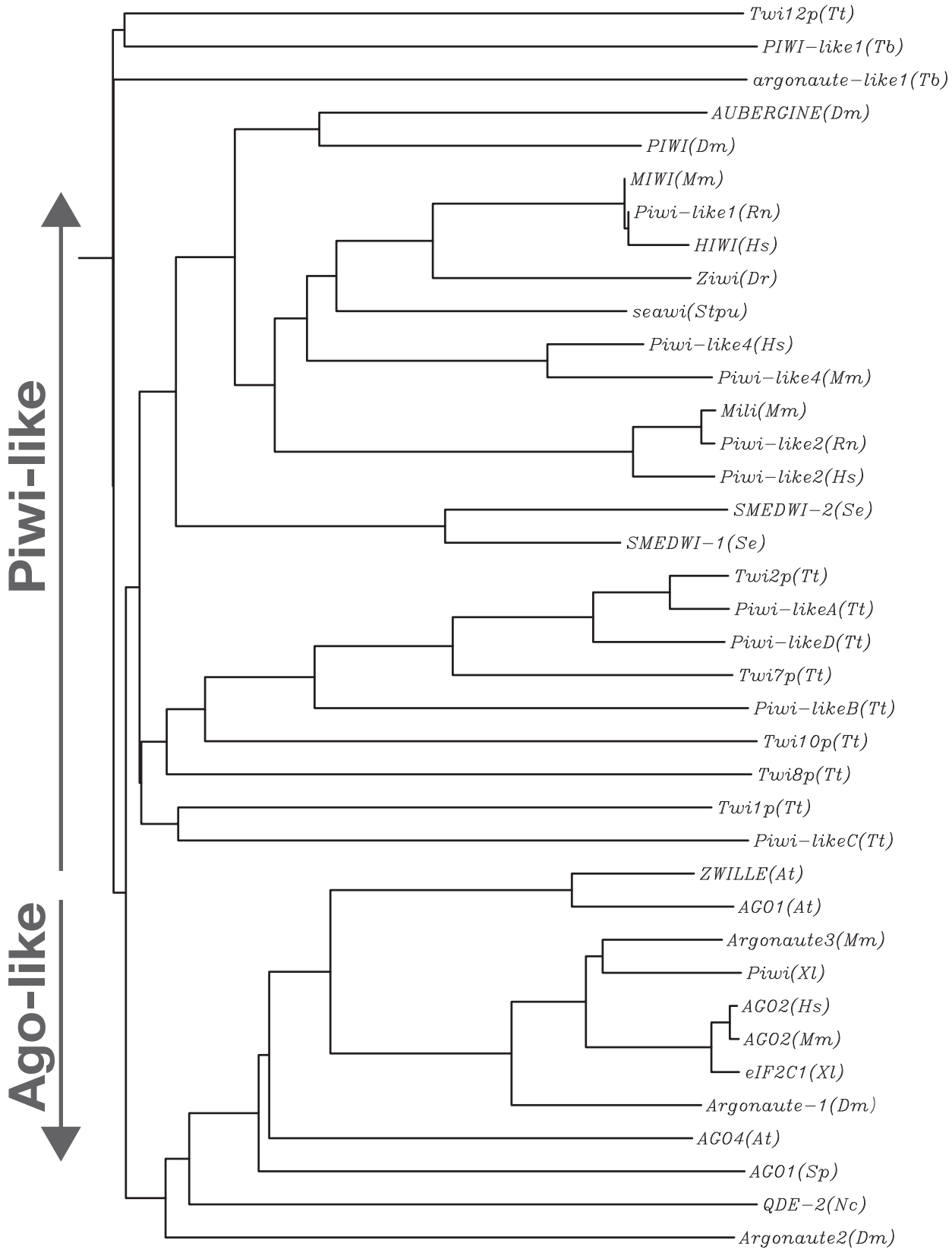

Twi12p(Tt)
PIWI-like1(Tb)
argonaute-like1(Tb)
AUBERGINE(Dm)
PIWI(Dm)
MIWI(Mm)
Piwi-like1(Rn)
HIWI(Hs)
Ziwi(Dr)
seawi(Stpu)
Piwi-like4(Hs)
Piwi-like4(Mm)
Mili(Mm)
Piwi-like2(Rn)
Piwi-like2(Hs)
SMEDWI-2(Se)
SMEDWI-1(Se)
Twi2p(Tt)
Piwi-likeA(Tt)
Piwi-likeD(Tt)
Twi7p(Tt)
Piwi-likeB(Tt)
Twi10p(Tt)
Twi8p(Tt)
Twi1p(Tt)
Piwi-likeC(Tt)
ZWILLE(At)
AGO1(At)
Argonaute3(Mm)
Piwi(Xl)
AGO2(Hs)
AGO2(Mm)
eIF2C1(Xl)
Argonaute-1(Dm)
AGO4(At)
AGO1(Sp)
QDE-2(Nc)
Argonaute2(Dm)
Piwi-like
Ago-like

Figure Legend I-1: Argonaute family of proteins. CLUSTALW branched tree analysis of Ago and Piwi protein subclades from human (Hs), mouse (Mm), rat (Rn), frog (Xl), fish (Dr), planaria (Se), fly (Dm), fission yeast (Sp), fungi (Nc) sea urchin (Stpu), trypanosome (Tb), ciliated protozoa (Tt), and plants (At).

Figure I-2

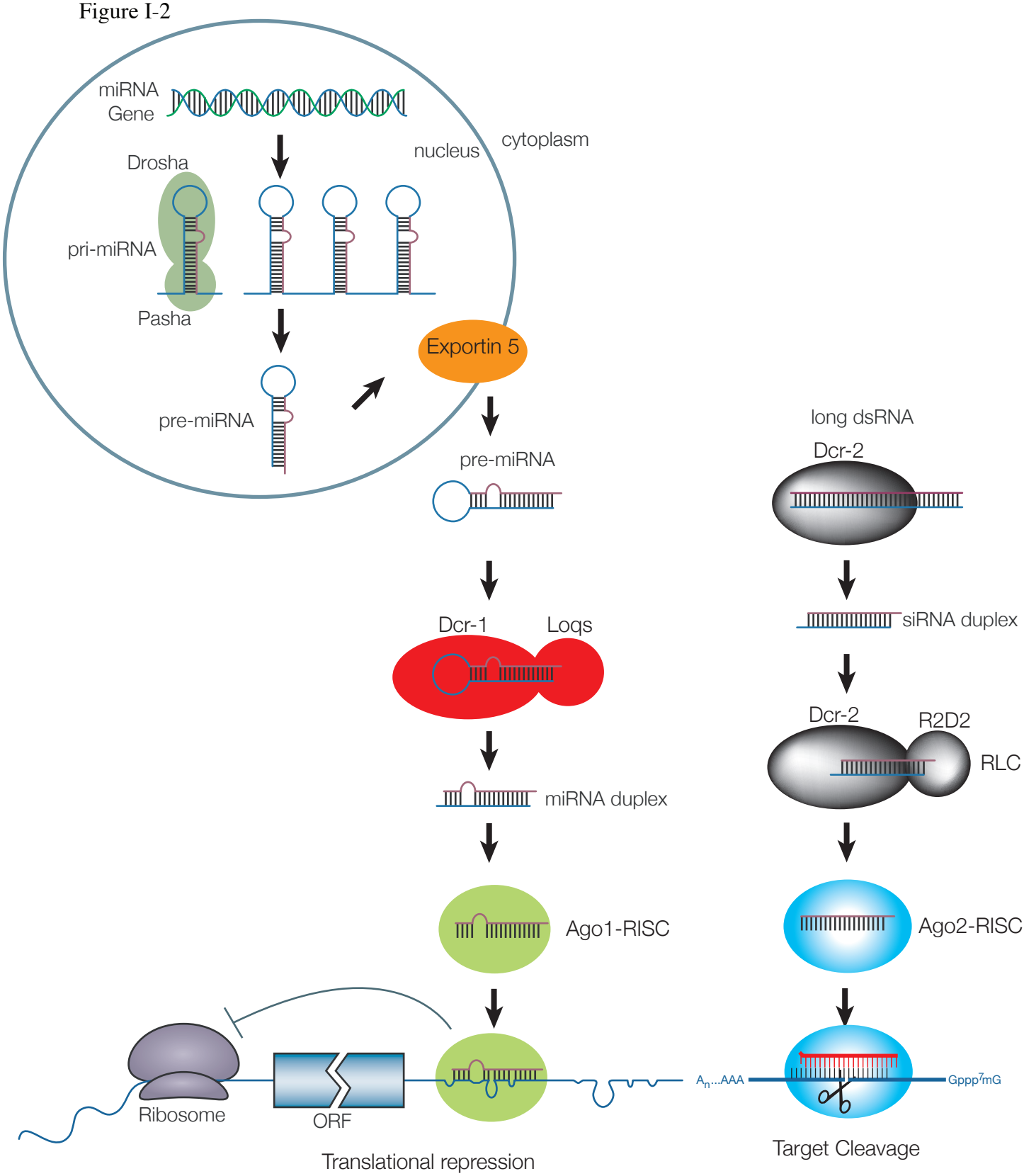

miRNA
Gene
nucleus
cytoplasm
Drosha
pri-miRNA
Pasha
pre-miRNA
Exportin 5
pre-miRNA
Dcr-1
Loqs
miRNA duplex
Ago1-RISC
Ribosome
ORF
Translational repression
long dsRNA
Dcr-2
siRNA duplex
Dcr-2
R2D2
RLC
Ago2-RISC
An...AAA
Gppp7mG
Target Cleavage

Figure Legend I-2: Biogenesis of miRNAs and siRNAs. 22 nucleotide long microRNAs (miRNAs, left) are encoded in their own genes. Pre-miRNAs are processed by Dicer into miRNA duplexes that are loaded into Argonaute protein by a mechanism which is unclear at this time. Small interfering RNAs (siRNAs, right) biogenesis in flies. Long double stranded RNA from a variety of sources (i.e. transgene or viral intermediates) are processed by Dicer into 21 nt duplex siRNAs. In flies, siRNAs are bound again by Dicer, and a double stranded RNA binding protein R2D2. This heterodimer—part of the larger RISC-loading complex (RLC)—is thought to initiate siRNA unwinding and loading of Argonaute 2 (Ago2) with the guide strand (red) of the siRNA duplex. (Adapted from He and Hannon, 2004, *Nat Rev Genet*)

processed first by the pre-mRNA splicing machinery in the nucleus, rather than Drosha, and then by Dicer in the cytoplasm (Okamura et al. 2007; Ruby et al. 2007).

miRNAs are an ancient innovation among animals: for example, both of the first two miRNAs discovered, *lin-4* and *let-7*, are conserved from nematodes to humans (Lee et al. 1993; Pasquinelli et al. 2000). The conservation of miRNAs between monocots such as wheat and dicots such as *Arabidopsis*—plants that diverged over two hundred million years ago—suggests an ancient origin for miRNAs in plants, too, and the recent discovery of miRNAs in the green unicellular alga, *Chlamydomonas reinhardtii*, hints that miRNAs could date back to the dawn of photosynthetic eukaryotes (Reinhart et al. 2002; Tang et al. 2003; Zhao et al. 2007; Molnar et al. 2007). Nonetheless, there is no compelling evidence that plants and animals share any miRNA in common, suggesting that miRNAs may have arisen at least twice in evolution, perhaps from an ancestral RNAi pathway.

Plant miRNAs are nearly perfectly complementary to the mRNAs they regulate, allowing them to direct the Argonaute protein, Ago1, to cleave their targets, which are often members of transcription factor families that regulate leaf or floral development (Bohmert et al. 1998; Rhoades et al. 2002; Llave et al. 2002; Baumberger and Baulcombe 2005). Both plant miRNAs and small interfering RNAs (siRNAs) are 2´-*O*-methylated at their 3´ termini by the *S*-adenosyl methionine–dependent methyltransferase, HEN1 (Yu et al. 2005). This modification is thought to protect plant small RNAs from 3´ polyuridylation, a signal likely to promote small RNA degradation (Li et al. 2005). Although animal miRNAs are generally not terminally modified, *Chlamydomonas*

miRNAs are, suggesting that the small RNA methylase, HEN1, was present in an ancestral, unicellular plant (Molnar et al. 2007).

siRNAs

siRNAs guide RNAi, a conserved eukaryotic response to foreign nucleic acids and the primary anti-viral defense for plants and some animals (Mourrain et al 2000; Wang et al. 2006). Since the discovery of siRNAs in 1999 by Hamilton and Baulcombe, subclasses of siRNAs have proliferated. Most are simply siRNAs with unusual functions, while others are not really siRNAs at all. By convention, the names of major classes of small silencing RNAs—miRNAs, siRNAs, and piRNAs—are not intended to encapsulate their regulatory mechanisms or biological functions, but rather to reflect their distinct modes of production. For simplicity, siRNAs will be subdivided into just two kinds: those that derive directly from the dsRNA trigger and function without amplification (Figure I-2, right), and siRNAs whose function requires amplification of an initial long double-stranded trigger RNA by an RNA-dependent RNA polymerase (RdRP; Figure I-3).

Canonical or primary siRNAs are produced from dsRNA by Dicer, which cleaves processively from one end of the dsRNA towards the other, moving in ~21 nucleotide steps (Bernstein et al. 2001; Lee et al. 2004; Zhang et al. 2004). Dicer substrate RNA can arise from viral replication intermediates, convergent transcription, transcripts that self anneal (hairpins), or experimentally introduced dsRNA (Hammond 2005). The product of dicing is the siRNA, a 21 nt duplex containing 19 base pairs and 2-nucleotide, 3´

overhanging ends (Zamore et al. 2000; Hammond et al. 2000; Bernstein et al. 2001). Dicer products contain 5´ phosphate and 2´,3´ hydroxy termini (Elbashir et al. 2001). Double-stranded siRNAs are assembled into Argonaute proteins by specific assembly factors that act to orient the siRNA within the Argonaute protein (Schwarz et al. 2003; Tomari et al. 2004b). siRNAs must be dissociated into their component single strands in order to act as guides for the protein complexes that repress gene expression (Nykänen et al., 2001; Martinez et al., 2002). In Argonaute, the 5´ end of single-stranded siRNA is anchored by a conserved phosphate-binding domain (Nykanen et al. 2001; Parker et al. 2005; Ma et al. 2005). Such single-stranded, Argonaute-bound siRNAs guide Argonaute proteins to cleave a single phosphodiester bond within the target RNA (Zamore et al. 2000; Hammond et al. 2001; Okamura et al., 2004; Meister et al., 2004; Rand et al., 2004; Liu et al., 2004; Rivas et al., 2005). This cleavage site is measured from the 5´ end of the siRNA guide, between the target nucleotides paired to siRNA nucleotides 10 and 11.

Plants, fungi, and some animals such as *C. elegans*, amplify the RNAi response by copying the target RNA into new siRNAs, known as secondary siRNAs (Dalmay et al. 2000; Mourrain et al. 2000; Motamedi et al. 2004; Sijen et al. 2001)(Figure I-3). These organisms encode RdRP enzymes that copy single-stranded RNA into complementary RNA. The RdRPs that function in RNAi generate secondary siRNAs by copying the mRNA targeted by primary siRNAs. Primary siRNAs were originally thought to prime the production of double-stranded RNA by the RdRP copying the target RNA

Figure I-3

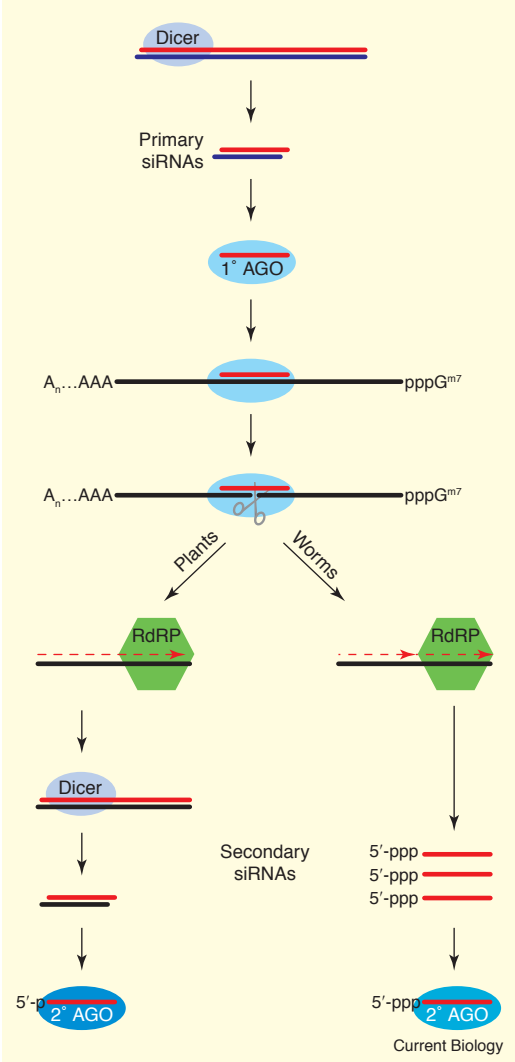


Figure Legend I-3: Amplification of the RNAi response by RNA-dependent RNA polymerases (RdRPs) in plants and worms. In plants, RdRP enzymes convert target RNAs to double stranded RNA that is cleaved by Dicer into secondary siRNAs. In worms, secondary small RNAs are short transcripts produced directly by RdRPs that use the target of the primary siRNA as a template (from Matranga and Zamore 2007, *Current Biology*).

into complementary RNA, which would then be diced into secondary siRNAs. A growing body of evidence argues against this mechanism. Instead, it appears that RdRPs can generate dsRNA by copying a target RNA end-to-end, without the use of primers, but that cleavage of the target RNA by primary siRNAs or miRNAs—probably to remove the cap and poly(A) tail of the target—is a prerequisite for the RdRP to use an RNA as a transcription template (Pak and Fire 2007; Sijen et al. 2007).

In plants, the new dsRNA is then diced processively to create a phased array of stereotypical siRNAs that regulate other mRNA targets in *trans*. This specific class of RdRP-dependent plant small RNAs—*trans*-acting siRNAs (tasiRNAs)—are produced when miRNA- or siRNA-guided Ago1 cleaves its target and the resulting RNA fragment is copied into double-stranded RNA substrate for dicing (Allen et al. 2005).

In *C. elegans*, the RNAi response is initiated by primary siRNA diced from a long, double-stranded RNA trigger (Knight and Bass 2001; Tabara et al. 2002). The resulting primary siRNAs trigger the copying of the RNA target into secondary siRNAs by an RdRP (Sijen et al. 2001). Worm secondary siRNAs—which are far more abundant than the initial primary siRNAs produced by dicing—are likely produced directly by transcription, without a dsRNA intermediate or dicing (Pak and Fire 2007; Sijen et al. 2007). These secondary siRNAs are exclusively antisense to the RNAi target, include sequences both upstream and downstream of the original dsRNA trigger, and begin with the 5′ di- or triphosphate group characteristic of transcription rather than the monophosphate that is the hallmark of dicing (Elbashir et al. 2001b).

Cleavage directed by the primary siRNAs may not be required for secondary siRNA production in *C. elegans*, as secondary siRNAs can be produced by a single primary siRNA only imperfectly complementary to its mRNA target (Sijen et al. 2007). Thus, the primary function of worm primary siRNAs may be to recruit an RdRP to the target RNA. Moreover, *C. elegans* secondary siRNAs may be loaded into specialized Argonaute proteins which bind multi-phosphate termini instead of the 5´ monophosphate found on canonical siRNAs (Pak and Fire 2006).

Plants produce siRNAs from inverted repeat transcripts through the action of two different Dicers. Dicer-like-4 converts dsRNA into canonical siRNAs, 21 nucleotides long, that target mRNAs for cleavage (Dunoyer et al. 2005). Dicer-like-3 makes ~24 nucleotide siRNAs, the most abundant class of siRNAs in plants, implicated in directing DNA and histone methylation (Xie et al. 2004; Lippman et al. 2003). All plant siRNA duplexes, both RdRP dependent and independent, like plant miRNA duplexes, are 2´-*O*-methylated at their 3´ termini by the HEN1 methyltransferase (Yu et al. 2005). *C. elegans* also has multiple classes of endogenous siRNAs. 21U-RNAs all begin with uracil and originate from only a few clusters that are specific to chromosome IV (Ruby et al. 2006). On this chromosome, each 21U-RNA is flanked by a bipartite upstream motif, suggesting that each arises from its own transcript. However, these small RNAs bear 5´ monophosphates, so perhaps their 5´ ends are created by endonucleolytic processing or the initial 5´ triphosphate is post-transcriptionally converted to a monophosphate. The 21U-RNA bipartite motif, but not any of the more than 10,000 individual 21U-RNA sequences, is conserved between *C. elegans* and *C. briggsae*. Thus 21U-RNAs likely act

in *cis* to regulate their own genomic locus. *C. elegans* also produces siRNAs 26 nucleotides long that always begin with guanosine and possess monophosphate 5′ ends (Ruby et al. 2006). Both the 21U- and 26-mer siRNAs have blocked 3′ termini. The function of 3′ terminal modification is not yet known for any animal small silencing RNA class. Another class of small RNA—tiny noncoding RNAs (tncRNA)—correspond to the antisense strands of protein coding genes, begin with a 5′ di- or triphosphate, and may be a form of endogenous secondary siRNA produced without dicing by RdRP-catalyzed transcription (Ambros et al. 2003).

piRNAs

PIWI-interacting RNAs (piRNAs), 24–30 nucleotide long RNAs found in the germ cells of animals, are unique among small silencing RNAs in that they require neither an RdRP nor Dicer for their production (Vagin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Aravin et al. 2006; Lau et al. 2006; Saito et al. 2006) (Figure I-4). Instead, they are thought to derive from single-stranded precursor RNAs tens or hundreds of thousands of nucleotides long. piRNAs bind a distinct subclade of Argonaute proteins, the PIWI proteins, which include Piwi, Aubergine, and Ago3 in flies, Smedwi in planaria, Seawi in sea urchins, and Hiwi, Hiwi2 and Hili in humans (Sharma et al. 2001; Sasaki et al. 2003; Reddien et al. 2005; Rodriguez et al. 2005; Brennecke et al. 2007). *In vitro*, Piwi can cleave a target RNA, suggesting that piRNAs regulate their targets post-transcriptionally, but other evidence suggests they promote heterochromatin assembly in the nucleus (Cox et al. 2000; Lau et al. 2006). piRNAs were first identified in 2001 in flies, where they

Figure I-4

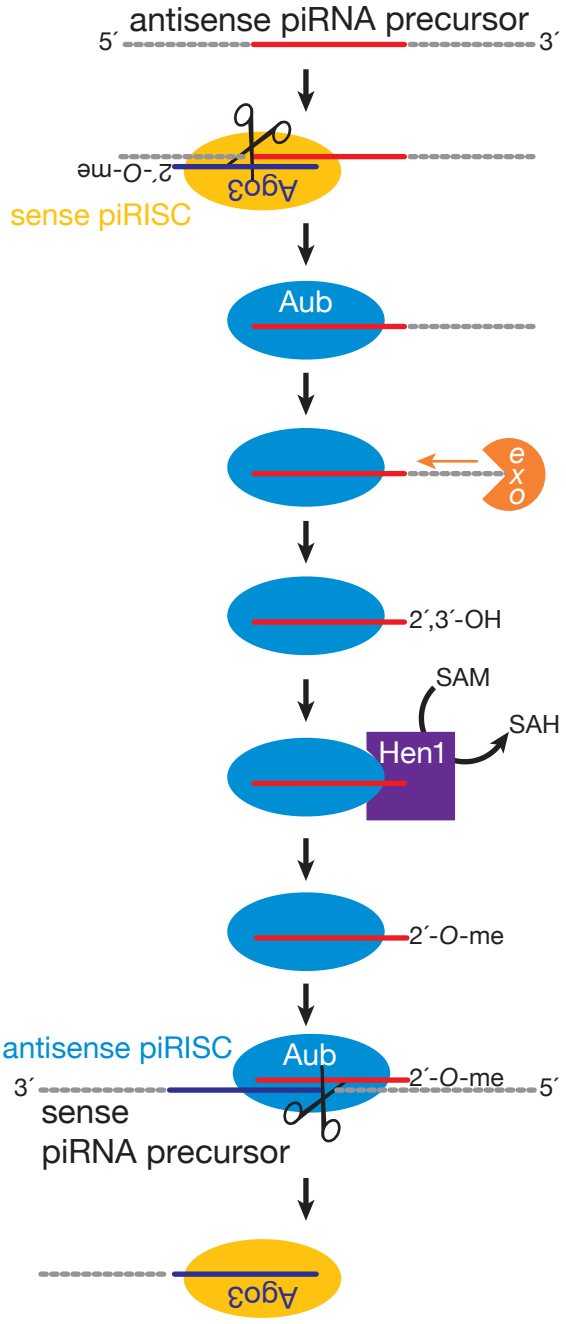


Figure Legend I-4: A speculative model for production of piRNAs in animal germ cells.

repress selfish genetic elements such as retrotransposons (Aravin et al. 2001). piRNAs contain 5′ monophosphate and 2′-*O*-methyl, 3′ hydroxy termini (Ohara et al. 2007; Kirino and Mourelatos 2007). In flies, piRNAs are thought to arise from "master loci" rich in transposons, then act in *trans* to silence dispersed copies of the selfish genetic elements present in the original trigger locus (Vagin et al. 2006; Brennecke et al. 2007). piRNAs have also been implicated in silencing transposons early in mammalian spermatogenesis (Aravin et al. 2007). In mammals, however, many piRNAs map to genomic clusters that do not contain repetitive sequences (Lau et al. 2006; Girard et al. 2006; Aravin et al. 2007). The functions of these piRNAs are unknown.

piRNAs may be generated by reciprocal cycles of PIWI-protein-catalyzed slicing followed by 3′ trimming by an exonuclease (Figure I-3). In *Drosophila*, for example, the first 10 nucleotides of many piRNAs bound to Aubergine, most of which are antisense to transposable element transcripts, can be paired to piRNAs associated with Ago3 (Brennecke et al. 2007; Gunawardane et al. 2007). Nearly all Aubergine-bound piRNAs begin with uracil, whereas Ago3-associated piRNAs, which are almost all in the sense orientation, typically contain an adenosine at nucleotide 10—reflecting their base pairing with the first nucleotide of an antisense piRNA. Hence the suggestion that piRNAs are amplified by reciprocal rounds of cleavage, in which Ago3 sense piRNAs direct cleavage of antisense transcripts producing the 5′ monophosphate end of Aub and Piwi antisense piRNAs. A 3′-to-5′ exonuclease could then trim the 3′ end of piRNA transcripts, perhaps acting together with the *Drosophila* piRNA methyltransferase, which might

terminate the trimming process by adding a 2´-*O*-methyl group to the 3´ terminus of the mature piRNA (Kirino et al. 2007a; Ohara et al. 2007).

Gene Regulation by Small RNAs

DNA elimination in *Tetrahymena*

In ciliated protozoa, *Tetrahymena thermophilus*, two nuclei exist, a transcriptionally-active somatic macronucleus, and dormant germline micronucleus (Figure I-5). It is thought that because the micronucleus can divide through meiosis, it acts as a control system to remove repetitive sequences from the macronucleus, which is eventually reassembled, but does not divide, during reproduction. Only during conjugation (sexual reproduction), the micronucleus genome is bidirectionally transcribed and the resulting dsRNA is processed by Dicer-like-1 (DCL1) into a subset of small RNAs 26 to 31 nucleotides long—scanRNAs (scRNAs) (Chalker and Yao 2001; Mochizuki and Gorovsky 2005). scRNAs "scan" the parental macronuclear genome and direct histone metylation (H3K9) at homologous, micronucleus-limited loci, marking the loci for internal DNA elimination in the developing macronucleus (Liu et al. 2004; Malone et al. 2005). scRNAs function requires the piwi-like protein, Twi1 (Mochizuki et al. 2002). A smaller class of RNAs (23-24 nts) were recently identified in *Tetrahymena*; the function of these RNAs is not known. These small RNAs show strand bias and require one of the three *Tetrahymena* Dicer-like proteins and an RdRP activity for biogenesis (Lee and Collins 2006).

Figure I-5

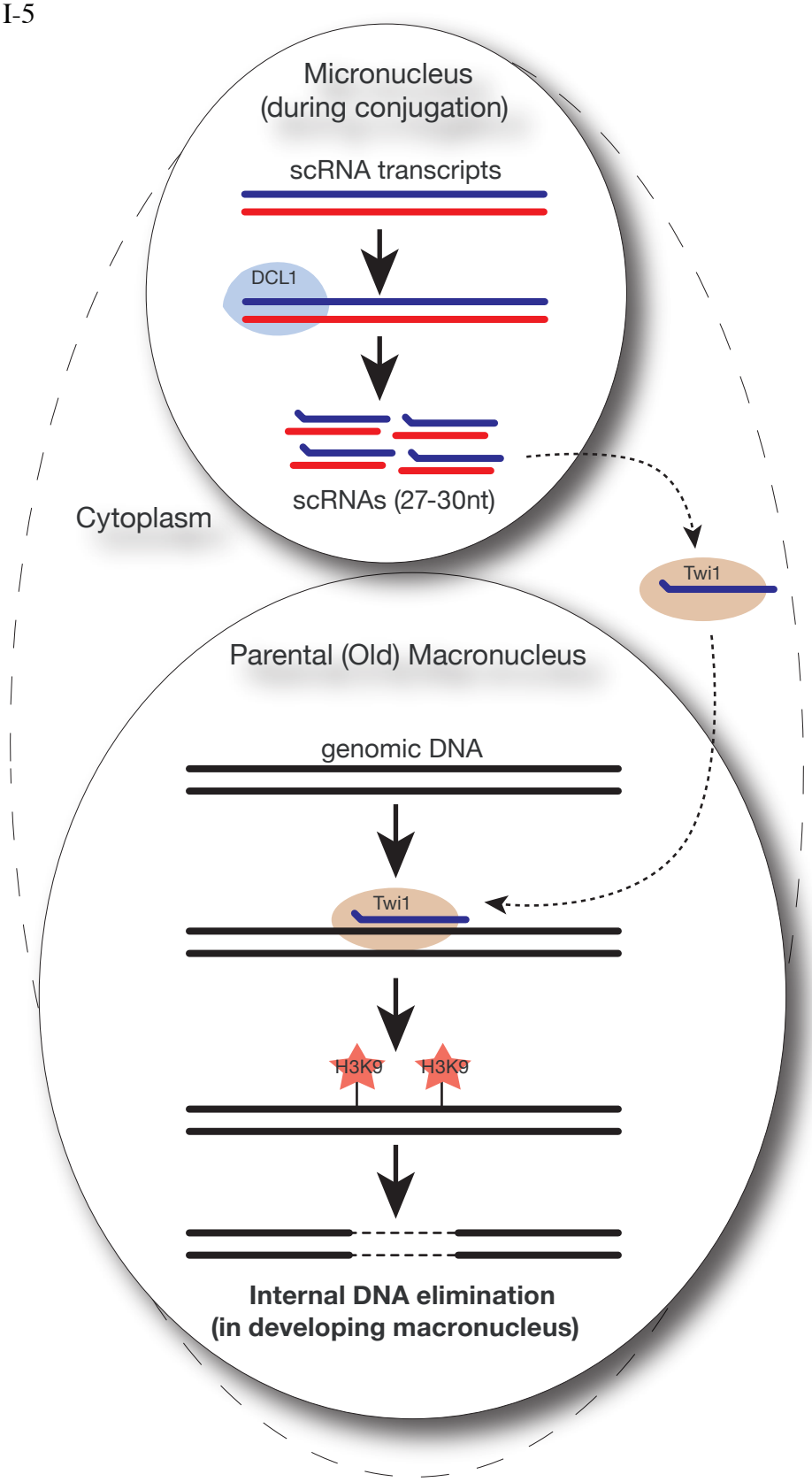

Micronucleus
(during conjugation)
scRNA transcripts
DCL1
scRNAs (27-30nt)
Cytoplasm
Twi1
Parental (Old) Macronucleus
genomic DNA
Twi1
H3K9
H3K9
Internal DNA elimination
(in developing macronucleus)

Figure Legend I-5: In *Tetrahymena thermophila*, 30 nt long small RNAs—scanRNAs (scRNAs)—are produced in the micronucleus during conjugative reproduction. scRNAs are programmed into Twi1, a homolog of *Drosophila* Aubergine and Piwi-clade Argonaute proteins, either in the cytoplasm or the parental macronucleus. Once in the parental macronucleus, scRNAs direct histone 3 methylation at lysine 9 (H3K9), setting the stage for subsequent internal elimination of micronucleus-specific DNA elements.

Transcriptional and Co-transcriptional gene silencing

Small RNAs can also direct heterochromatin formation, silencing gene at the transcriptional level. In the fission yeast, *Schizosaccharomyces pombe*, siRNAs can regulate gene expression both post-transcriptionally and co-transcriptionally (Volpe et al. 2002; Hall et al. 2002; Buhler et al. 2006). The source of dsRNA from which *S. pombe* siRNAs are diced is thought to be RNA polymerase II transcripts converted to dsRNA by the RdRP, Rdp1p, which is a component of a complex that also contains a putative RNA helicase and a nucleotidyl transferase (Kato et al 2005; Sugiyama et al. 2005; Motamedi et al. 2004). The resulting siRNAs are thought to recruit an Argonaute-protein complex to nascent transcripts, directing the deposition of repressive chromatin marks, such as histone 3 lysine 9 methylation (H3K9), on centromeric DNA repeats (Verdel et al. 2004; Motamedi et al. 2004; Buhler et al. 2006).

Similar siRNA-directed nuclear silencing phenomena have also been studied in detail in plants. Plant Dicer-like 3 produces a longer (24 nt) class of duplexes whose accumulation depends on HEN1, RNA dependent RNA polymerases, and plant-specific DNA-dependent RNA polymerases (Xie et al. 2004; Herr et al. 2005; Onodera et al 2005). These 24 mers bind Argonaute 4 (Ago4) and are thought to target either nascent transcripts or DNA, directing DNA methylation at specific loci (Zilberman et al. 2003; Xie et al. 2004).

Transcriptional silencing directed by small RNAs may also occur in animals. In *C. elegans*, germline small RNAs silence Tc1 transposons—loci from which they are produced—in a manner dependent on RNAi components (Sijen and Plasterk 2003).

Argonaute-dependent transcriptional-silencing was also found in somatic tissue of *C. elegans*; however, there was no direct evidence of heterochromatin formation or stable epigenetic loss of function (Grishok et al. 2005). In *Drosophila*, gene expression is often blocked at the transcriptional level by position effect varigation (PEV)—loci that is normally euchromatic is juxtaposed by heterochromatin formation (Grewal and Elgin 2007). Mutations that suppress PEV are often linked to heterochromatin formation and decreased levels of H3K9 methylation (Pal-Bhandra et al. 2002). As such, mutations in genes involved in siRNA and piRNA biogenesis and function including *piwi*, *aubergine*, and *spindle-E*—an RNA helicase—result in loss of *white* transgene silencing within heterochromatin, suggesting that small RNAs may promote TGS if stable repression of the target RNA is required (Pal-Bhandra et al. 2004). Loss of function *piwi* mutants in mice resulted in activation of transposons with a corresponding loss of DNA methylation, suggesting that piRNAs may direct DNA methylation (Aravin et al. 2007; Carmell et al. 2007). Although there is no direct evidence, these data suggest piRNAs regulate expression of transposable elements in higher vertebrates by directing sequence-specific heterochromatin formation.

Post Transcriptional Gene Silencing

siRNAs generally always reduce gene expression by traditional post-transcriptional silencing or RNAi. siRNA function is best understood for flies and mammals, where they guide the Argonaute2 protein to cleave complementary target RNAs at the phosphodiester bond across from siRNA nucleotides 10 and 11, leaving a 5´

fragment with a 3´ hydroxy and a 3´ fragment with 5´ phosphate terminus (Schwarz et al. 2003; Figure I-2). Some siRNAs—chiefly those with mismatches engineered in the central region to mimic miRNA:target duplexes—act by controlling an mRNA's translation (Doench et al. 2003). siRNAs can also function by activating transcription or translation of mRNA by an unknown mechanism that relies on specific Argonaute proteins and cofactors (Li et al. 2006; Vasudevan and Steitz 2007).

Translational control by miRNPs

Animal miRNAs are typically less complementary to their target mRNAs than those in plants (Bartel 2004). While animal miRNAs can direct the Argonaute protein Ago2 to cleave an mRNA target—a process that requires extensive base pairing between the miRNA and its binding site on the mRNA—this form of regulation is rare with only a few examples identified in mammals among the tens of thousands of predicted miRNA:mRNA regulatory pairs (Yekta et al. 2004). Instead, most animal miRNAs either block mRNA translation or target mRNAs for destruction by standard mRNA turnover mechanisms.

miRNAs can bind their targets even when they are complementary at just six—very special—contiguous bases. This region of the miRNA, comprising miRNA nucleotides 2 through 7, is known as the "seed," and is created only when the miRNA is fully engaged with an Argonaute protein (Lai 2002; Lewis et al. 2003; Brennecke et al. 2005). The ability of miRNAs to bind their targets through the seed alone makes the experimental finding of targets difficult, although computational predictions have been

surprisingly successful, especially when evolutionary conservation of miRNA-binding sites is used to evaluate preliminary results (Lewis et al. 2005).

How miRNAs regulate their mRNA targets remains contentious. Do some miRNAs increase mRNA turnover while others repress translation, or is mRNA instability a consequence of blocking translation for some mRNAs? Which step of translation do miRNAs regulate? Some data suggest that miRNAs block protein translation after the initiation of translation, perhaps by inhibiting polypeptide elongation or even by degrading the nascent peptide (Petersen et al. 2006; Nottrott et al. 2006). Recent data implicate translational initiation as the regulated step. In human cells, Ago2 has been proposed to contain a domain that binds the 7-methyl-guanosine cap of mRNA, competing with the binding of eIF4E that is required to recruit ribosomes to a message (Kiriakidou et al. 2007). Alternatively, miRNAs have been hypothesized to increase the concentration of eIF6 on their target mRNAs, thereby antagonizing ribosome subunit joining, a prerequisite for the assembly of a functional ribosome (Chendrimada et al. 2007). Animal miRNPs have also been proposed to direct mRNAs to cytoplasmic sites of RNA degradation such as P-bodies or stress granules (reviewed in Valencia-Sanchez et al. 2006). In zebrafish, miRNAs promote deadenylation of maternal RNA poly(A) tails at the onset of zygotic transcription, a clever way of clearing unneeded transcripts that might otherwise sequester much-needed ribosomes (Giraldez et al. 2006). miRNA-directed deadenylation has also been observed in cultured mammalian cells (Wu et al. 2006).

The RNA Interference Pathway in *Drosophila melanogaster*

Initiation and Intermediates of RISC assembly

The RNAi pathway has been well established through a combination of genetic and biochemical experiments in the model system *Drosophila melanogaster*. First, long double stranded RNA is processed by the ribonuclease III, Dicer2 into 21nt-long siRNAs (Hammond et al. 2001; Lee et al. 2004). Importantly, the Tuschl group discovered that introduction of siRNAs in human cell culture and in *Drosophila* embryo lysate bypass the initial dicing step and mediate silencing of RNA (Elbashir et al. 2001a; Elbashir et al. 2001b; Nykanen et al. 2001), allowing siRNAs to be used as a genetic tool to knockdown expression of any gene as well as substrates to dissect the RNAi mechanism.

siRNA duplexes, like some miRNA duplexes, are functionally-asymmetric; the thermodynamic stability of each 5´ end of the siRNA determines which strand of the duplex loads RISC (Khvorova et al. 2003; Schwarz et al. 2003). In *Drosophila melanogaster*, dissociation of the two siRNA strands requires the Dicer-2 (Dcr-2)/R2D2 protein heterodimer, as well as Ago2—the core component of RISC—itself (Liu et al., 2003; Lee et al., 2004; Pham et al., 2004; Okamura et al., 2004). Dcr-2 and R2D2 act in the RISC-loading complex (RLC) to sense the relative local stabilities of the 5´ ends of each siRNA strand, preferentially loading into Ago2 the siRNA strand less tightly base paired at its 5´ end (Tomari et al., 2004b; Figure I-2). The strand that is programmed into RISC is called the guide strand because it "guides" Ago2-RISC to its complementary target (Schwarz et al. 2002). The other strand of the siRNA duplex, the passenger strand, is destroyed during RISC assembly. In *Drosophila* embryo lysate lacking Ago2, siRNAs

remain double-stranded (Okamura et al., 2004) and stably bound to Dcr-2 and R2D2 (Tomari et al., 2004b), suggesting that siRNA strand separation is initiated only after Ago2 interacts with the siRNA bound to Dcr-2 and R2D2 or that an intermediate complex exists that contains both Ago2 and double stranded siRNA.

In lysates prepared from *Drosophila* S2 cells, synthetic siRNA was loaded into an 80S complex made up of Dcr-1—the *Drosophila* Dicer involved strictly in miRNA biogenesis—as well as Dcr-2, R2D2, Fragile-X protein (DmFXR), a staphylococcal nuclease, and Vasa Intronic Gene (VIG)(Pham et al. 2004) (Table 1). DmFXR was also found in a complex with miRNAs, Ago2, and ribosomal proteins (Ishizuka et al. 2002). The functions of the 80S complex and associated proteins are unclear; however, it was postulated that the complex may be a large "assemblosome" present in *Drosophila*, capable of loading both miRNAs and siRNAs.

Armitage (Armi) is a putative helicase that contains homology to SDE3, a helicase required for transgene PTGS in plants (Dalmay et al. 2001; Cook et al. 2004). Armi may function during RISC assembly in flies (Cook et al. 2004; Tomari et al. 2004a); however, the function of Armi or how it promotes complex formation is currently unknown. Moreover, the helicase proposed to unwind siRNA duplexes during RISC assembly has yet to be discovered (Nykanen et al. 2001). RNA exonucleases may be closely associated with RISC activity, most likely initiating the metabolism of RISC-cleaved mRNAs (Orban and Izaurralde 2005).

Table 1. Potential players in the *Drosophila* RNAi pathway

Gene name	Putative function	Homologs in other organisms?
Dicer-2	siRNA production; RISC loading	Dicer proteins
Dicer-1	siRNA production	Dicer proteins
R2D2	dsRNA binding; RISC loading	Hyl1 (At), RDE4 (Ce), TRBP (Hs)
Loquacious	dsRNA binding; miRNA processing; siRNA reservoir?	Hyl1 (At), RDE4 (Ce), TRBP (Hs)
Argonaute2	Single-strand RNA cleavage; RISC assembly; siRNA-RISC	Argonaute proteins
Argonaute1	Single-strand RNA cleavage; miRNA-RISC	Argonaute proteins
Aubergine	Single-strand RNA cleavage; RISC assembly	Piwi proteins
Armitage	Putative DEAD box/RNA helicase	SDE3 (At), Mov10 (Hs)
Spindle-E	Putative DEAD box/ RNA helicase	No
Dmp68	Putative DEAD box/ RNA helicase	p68 (Hs)
Maleless	Putative RNA helicase	RNA helicase A (Hs)
Maelstrom	Putative HMG box helicase	Mael (Mm)
Tudor-SN (TSN)	Staphylococcal-like nuclease	TSN (Ce)
DmFXR1	KH-domain (RNA binding)	Fragile-X proteins (Hs)
Ski proteins	3′-5′ exonuclease	Exosome proteins (mammals)
Pacman	5′-3′ exonuclease	XRN1 (Hs), XRN4 (At)
Vasa Intronic Gene (VIG)	Unknown	No
Table Key: At, *Arabidopsis thaliana*; Ce, *Caenhorbaditis elegans*; Mm, *Mus musculus*; Hs, *Homo sapiens*		

Dissection of the ATP requirements provides insight into the mechanism of assembly of RNAi-RISC (Zamore et al. 2000; Nykanen et al. 2001; Pham et al. 2004; Tomari et al. 2004a). In early *Drosophila* embryo lysates, processing of long dsRNA into siRNAs requires ATP (Zamore et al. 2001). In the same lysates, 5´ phosphorylation of the siRNA, siRNA strand separation, and assembly of single stranded siRNA into RISC all require ATP (Nykanen et al. 2001). The need for ATP in complex formation is also known; binding of Dicer-2 and R2D2 to an siRNA does not require ATP; however, production of the larger RLC does require ATP (Pham et al. 2004; Tomari et al. 2004a). Overall, ATP hydrolysis is required for siRNA and RISC production in *Drosophila*.

Argonaute2-RISC

Argonaute 2 (Ago2) was initially identified as a member of RISC (Hammond et al. 2001) and is now known to be the core component of RISC in *Drosophila melanogaster* (Rand et al. 2004). Fly and mammal Ago2 contain an RNaseH-like endonuclease activity (Liu et al. 2004; Rand et al. 2005). Through Watson and Crick base pairs between guide and target RNA, Ago2-RISC cleaves at the phosphodiester linkage across from the 10^{th} and 11^{th} nucleotides of the guide (Martinez et al. 2002). At least one turn of an A-form helix—11 nucleotides—is required for Ago2 catalysis (Haley and Zamore 2004). Ago2 catalysis (i.e. target RNA cleavage) does not require ATP; however, Ago2 is a multiple turnover enzyme, capable of multiple rounds of target cleavage and release in the presence of an ATP-dependent cofactor (Hutvagner and Zamore, 2002; Haley and Zamore 2004; Rivas et al. 2005). Ago2 may also promote

target recognition by binding nonspecifically to single stranded RNA, enabling RISC to find and anneal—through the 5´ end of its guide—its target RNA (Ameres et al. 2007).

Structural studies of Argonaute proteins have elucidated the mechanism and assembly of RISC. Argonaute proteins have four domains: the N-terminus, PAZ, Mid, and PIWI domains (Tolia and Joshua-Tor 2007) (Figure I-6). The PAZ domain of *Drosophila* Ago2 recognizes and binds the 2 nt-3´ overhang of siRNAs (Lingel et al. 2003), a potential intermediate step in the assembly of Ago2-RISC (Tomari et al. 2004b; Yuan et al. 2006). The Mid domain of Argonaute proteins has a conserved phosphate-binding pocket, the docking site of the obligatory 5´phosphate group of the siRNA guide strand (Nykanen et al. 2001; Parker et al. 2005; Ma et al. 2005). The Argonaute PIWI domain has two functions: yeast two-hybrid analysis showed that RNaseIII domain of Dicer interacts with the PIWI domain of Ago proteins suggesting that Dicer may position Ago2 properly on the siRNA for RISC loading (Tahbaz et al. 2004; Tomari et al. 2004b). Structural analysis of archeal Argonautes showed that the PIWI domain adopts an RNaseH-like fold (Song et al. 2004). Mutations of the predicted catalytic residues in fly and human Ago2 suggested that the PIWI domain contains the "slicing" activity necessary for RISC mediated target cleavage (Liu et al. 2004; Rand et al. 2004).

Significance of RNAi

There are clear examples of the biological significance and overall importance of RNAi. As a tool, RNAi—or the use of siRNAs and other small RNAs—allows scientists asking questions about the significance or requirement of a specific gene to perform

Figure I-6

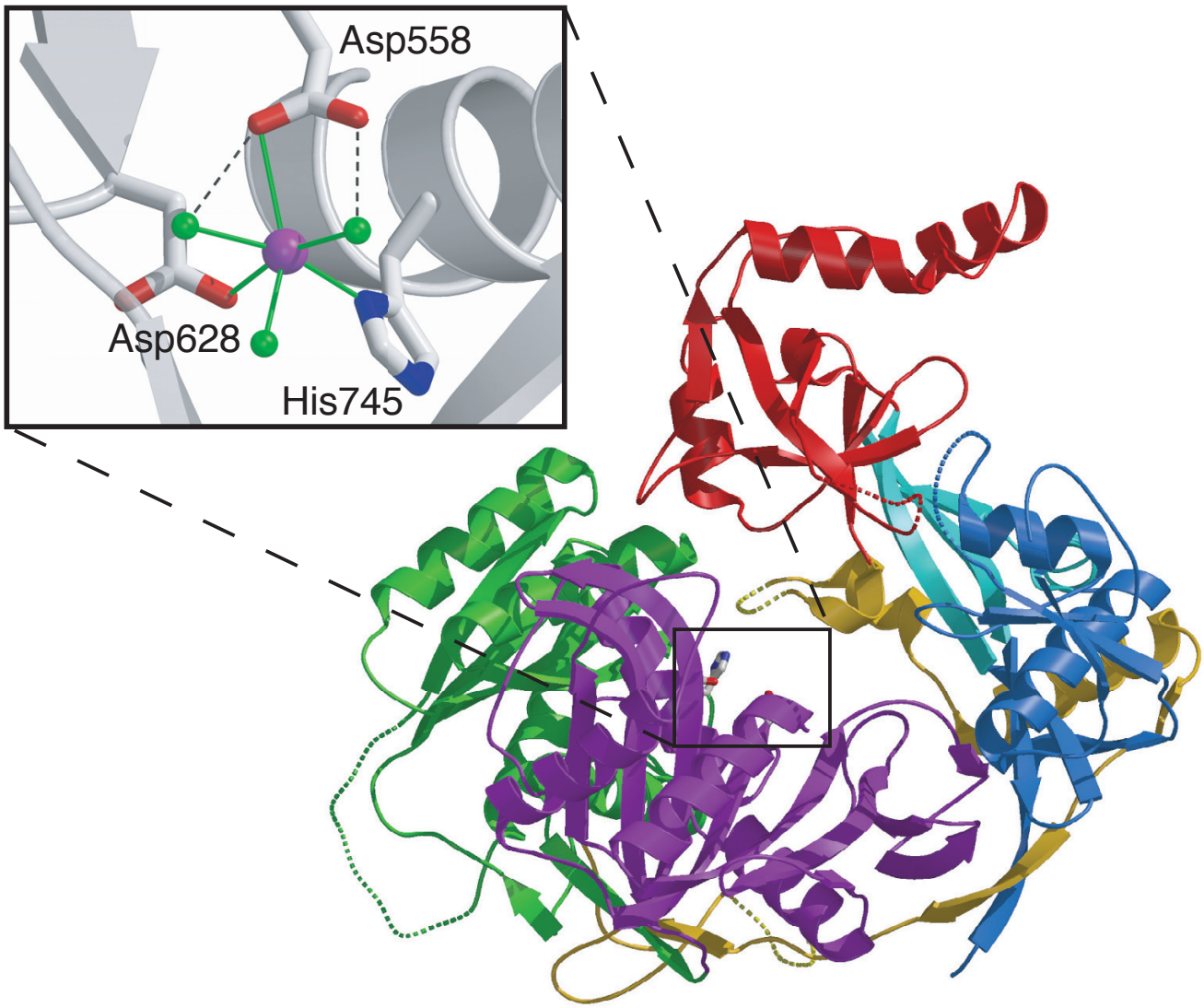


Figure Legend I-6: The crystal structure of Argonaute from *Pyrococcous furiosus*. The N-terminal domain is blue, the PAZ domain is red, the Mid domain is green, and the PIWI domain is purple. The PAZ domain is held above the crescent-shaped base created by the N-terminal, Mid, and PIWI domains. A closeup of the active site residues (Asp-Asp-His) coordinating a Mn2+ is shown (top left). Mn2+ was used in the crystallization to characterize the active site, as it easier to distinguish crystallograpraphically and can functionally replace Mg2+, the natural ion that is coordinated by the Ago Asp-Asp-His motif (from Tolia and Joshua Tor 2007, *Nature Chemical Biology*).

straighforward loss-of-function analysis. This discovery has revolutionized the field of biology, allowing scientists to pick specific targets for siRNA-knockdown and to score phenotypes much more rapidly than traditional genetic approaches. Biologically, RNAi is a conserved mechanism of foreign nucleic acid removal. In plants, PTGS by small RNAs is required to establish an innate immune response against viruses of which produce dsRNA during replication (Waterhouse et al. 1998; Hamilton and Baulcombe 1999). In *C. elegans*, viral replication is potentiated in cells and animals containing mutations in the Argonaute, RDE-1, and double-stranded RNA binding protein, RDE-4, both of which are required for RNAi in worms (Lu et al. 2005; Schott et al. 2005; Wilkins et al. 2005). Likewise, *dcr-2* and *r2d2* adult flies are vulnerable to viral infection suggesting that the RNAi mounts an innate immune response in animals (Wang et al. 2006). *ago2* mutant adult flies—although viable—are extremely sensitive to viral infections; virus-infected *ago2* flies show increased viral intermediates and titer compared to wild-type controls, further implicating RNAi as the primary innate immunity against viruses in *Drosophila* (van Rij et al. 2006). Unlike invertebrates, jawed vertebrates contain elaborate protein-based innate immune systems including interferon recognition of long dsRNA (Mak 2005). However, vertebrate organisms are thought to have once used RNAi to combat viruses. Many vertebrate-specific viruses produce miRNAs that are thought to squelch the components of the siRNA pathway, dampening the RNAi response as a counterdefense mechanism (Pfeffer et al. 2004). We await direct evidence of a vertebrate system that uses its RNAi machinery as an innate immune response against foreign nucleic acids.

Ago2 is the core component of siRNA-programmed RISCs in flies and mammals (Hammond et al. 2001; Liu et al. 2004; Rand et al. 2004). In these animals, mutations in Ago2 can lead to severe defects during development. In *Drosophila*, *ago2* null flies are viable, yet these animals display adverse developmental phenotypes (Deshpande et al. 2005; Meyer et al. 2006). During early embryogenesis, *ago2* flies show defects in syncytial nuclear division and cortical migration of the nuclei (Deshpande et al. 2005). Moreover, these defects are thought to lead to aberrant mitotic cell division and disrupted pole cell formation. Ago2 is essential for mouse development; Ago2 -/- mice display neural tube closure and heart failure due to enlarged cardiac muscle (Liu et al. 2004).

Small double stranded RNAs are also used as a theurapeutic tools in the clinic, targeting diseases such as macular degeneration as well as human viral infections caused by respiratory syncytial virus (RSV) (Bumcrot et al. 2006). An ultimate goal of many basic and clinical researchers studying RNAi is to obliterate dominant genetic disorders such as amyotrophic lateral sclerosis (ALS) and Huntingtin's disease. Recent studies were done *in vitro* and in cells that suggest siRNAs can discriminate between the mutant copy of the superoxide dismutase 1 gene, involved in ALS progression, and its normal copy—a difference of only a single nucleotide in some cases (Schwarz et al. 2006). One setback in the use of siRNAs as a theurapeutic is the potential of "off-target" effects that may lead to toxicity *in vivo* (Jackson and Linsley, 2004; Svoboda 2007). Expression of liver-specific short hairpin RNA (shRNA) constructs *in vivo* led to a high level of toxicity in mice, generally resulting in high levels of fatality (Grimm et al. 2006). Other issues in

the use of siRNAs in the clinic include the stability and delivery of siRNAs to their targets, issues all of which are currently under investigation (Bumcrot et al. 2006).

CHAPTER II: Plant RNAi *in vitro*

In the RNA interference (RNAi) pathway, small RNAs (siRNAs) direct the sequence-specific silencing of complementary RNA. RNAi and similar post-transcriptional gene silencing phenomena are found in other eukaryotes including animals, fungi, protozoa, and plants (Cogoni et al. 1996; Fire et al. 1998; Kennerdell and Carthew 1998; Ngo et al. 1998; Timmons and Fire 1998; Waterhouse et al. 1998; Vaucheret et al. 1998; Lohmann et al. 1999; Sánchez-Alvarado and Newmark 1999; Wianny and Zernicka-Goetz 2000; Caplen et al. 2001; Elbashir et al. 2001a; Volpe et al. 2002; Schramke and Allshire 2003). The RNase III endonuclease, Dicer, initiates RNAi by converting long, double-stranded RNA into small interfering RNAs (siRNAs) (Zamore et al. 2000; Bernstein et al. 2001; Billy et al. 2001), ~22-nucleotide guides that direct mRNA cleavage as components of a protein-RNA complex, the RNA-induced silencing complex (RISC) (Hamilton and Baulcombe 1999; Hammond et al. 2000; Elbashir et al. 2001b; Hammond et al. 2001). In *Drosophila melanogaster*, another protein-RNA complex, the RISC loading complex (RLC), assembles one of the two strands of an siRNA into the RISC. The RLC comprises double-stranded siRNA, a heterodimer of the RNase III endonuclease Dicer-2 (Dcr-2) and the siRNA-binding protein R2D2, and other yet identified components (Liu et al. 2003; Tomari et al. 2004a; Pham et al. 2004). Assembly of functional RISC also requires Armitage, a homolog of the *Arabidopsis thaliana* protein SDE3, a putative helicase required for RNA silencing triggered by sense RNA-expressing transgenes (Dalmay et al. 2001). Armitage is

required for oogenesis and RNA interference *in vivo* (Cook et al. 2004); biochemical evidence suggests that Armitage acts after the RLC in RISC assembly (Tomari et al. 2004a). Argonaute family proteins form the catalytic core of the RISC (Hammond et al. 2001; Tabara et al. 2002; Caudy et al. 2002; Hutvágner and Zamore 2002; Martinez et al. 2002; Mourelatos et al. 2002; Hutvágner et al. 2004; Song et al. 2004; Liu et al. 2004; Martinez and Tuschl 2004; Meister et al. 2004; Parker et al. 2004; Rand et al. 2004) and are thus required genetically for RNAi (Tabara et al. 1999; Fagard et al. 2000; Grishok et al. 2000; Catalanotto et al. 2002; Caudy et al. 2002; Morel et al. 2002; Pal-Bhadra et al. 2002; Williams and Rubin 2002; Doi et al. 2003). Transfer of the siRNA guide strand, the strand complementary to the target RNA, from the RLC to the RISC requires Argonaute 2, consistent with its proposed role as an acceptor of unwound siRNA guide strand (Okamura et al. 2004; Tomari et al. 2004b).

Extracts from *Drosophila* and mammalian cells are important tools for dissecting the mechanism of RNAi *in vitro*, yet no comparable *in vitro* system is available for plant RNAi initiated by siRNAs. In plants, wheat germ extract is an important tool for studying RNA silencing, but wheat germ extracts cannot be programmed with synthetic duplex siRNAs. Wheat extracts recapitulate some RNA silencing activities, including RNA-dependent RNA polymerase activity and small RNA production from long dsRNA (‘dicing’) (Tang et al. 2003). Wheat germ extract also contains functional RISCs programmed with microRNAs, small, endogenous RNA guides that control the expression of mRNA targets, typically by cleaving them, suggesting that the extracts are specifically defective in RISC assembly (Tang et al. 2003; Mallory et al. 2004).

Exogenous siRNAs trigger target cleavage activity in *Drosophila* embryo lysates and extracts from cultured mammalian cells (Elbashir et al. 2001b; Nykänen et al. 2001) (Boutla et al. 2001; Elbashir et al. 2001a), but not wheat germ extracts (Figure 1A). The siRNAs produced in *Drosophila* lysate are both double- and single-stranded (Nykänen et al. 2001) (Tang et al. 2003), but those produced in wheat germ extract remain double-stranded (Tang et al. 2003), suggesting that synthetic siRNA duplexes do not trigger RNAi in wheat germ extract because they are not unwound.

Here, we demonstrate that wheat germ extract cannot be programmed with exogenous siRNA duplexes because they do not unwind siRNA. In contrast, single-stranded siRNAs directed endonucleolytic cleavage of a corresponding target RNA in wheat germ extract. Wild-type *Drosophila* embryo and ovary lysate complemented the defect in siRNA unwinding in wheat germ extract. *Drosophila* ovary lysate defective in RNAi but not siRNA unwinding also rescued wheat germ extract, whereas mutant lysate defective for siRNA unwinding did not. Biochemical complementation of wheat germ RNAi requires both components of the RISC loading complex (RLC) and core components of mature *Drosophila* RISC.

Results

Wheat germ extract cannot unwind siRNA

Double-stranded siRNAs direct target cleavage in *Drosophila* embryo lysate (Boutla et al. 2001; Elbashir et al. 2001a; Elbashir et al. 2001c; Nykänen et al. 2001), but triggered only low levels of target cleavage in wheat germ extract (Figure 1A). We asked

if the failure of double-stranded siRNAs to trigger efficient target cleavage in wheat germ extract might reflect a defect in unwinding siRNA duplexes or in assembling the unwound strands into RISC. We monitored siRNA unwinding in wheat germ extract in the presence of ATP; in *Drosophila* embryo lysate, siRNA unwinding requires ATP (Nykänen et al. 2001). Wheat germ extract, unlike *Drosophila* embryo lysate, did not unwind double-stranded siRNAs: no single-stranded siRNA accumulated (Figure 1B) nor did the double-stranded siRNA decrease with time (data not shown). We conclude that wheat germ extract lacks a robust siRNA unwinding activity.

siRNA must contain a 5´ phosphate group to function in RNAi; blocking siRNA 5´ phosphorylation inactivates the siRNA (Nykänen et al. 2001; Chiu and Rana 2002; Schwarz et al. 2002). We examined whether the lack of siRNA unwinding in wheat germ extract was caused by the absence of an siRNA kinase. We incubated a 3´ ^{32}P-radiolabeled siRNA bearing a 5´ hydroxyl group in wheat germ extract and monitored its phosphorylation. The siRNA was rapidly phosphorylated in the wheat germ extract, as evidenced by its faster electrophoretic mobility (Figure 1C). Thus, wheat germ extract contains an siRNA kinase.

If siRNA unwinding, rather than siRNA phosphorylation, is defective in wheat germ extract, then single stranded, but not double-stranded, siRNAs might direct target mRNA cleavage in the extract. In vitro, in both *Drosophila* embryo lysates and extracts of cultured mammalian cells, and ex vivo, in cultured mammalian cells, single-stranded siRNAs act as guides for endonucleolytic cleavage, albeit with reduced efficiency (Schwarz et al. 2002). Figures 1D and 1E shows that single-stranded siRNAs also direct

Figure II-1

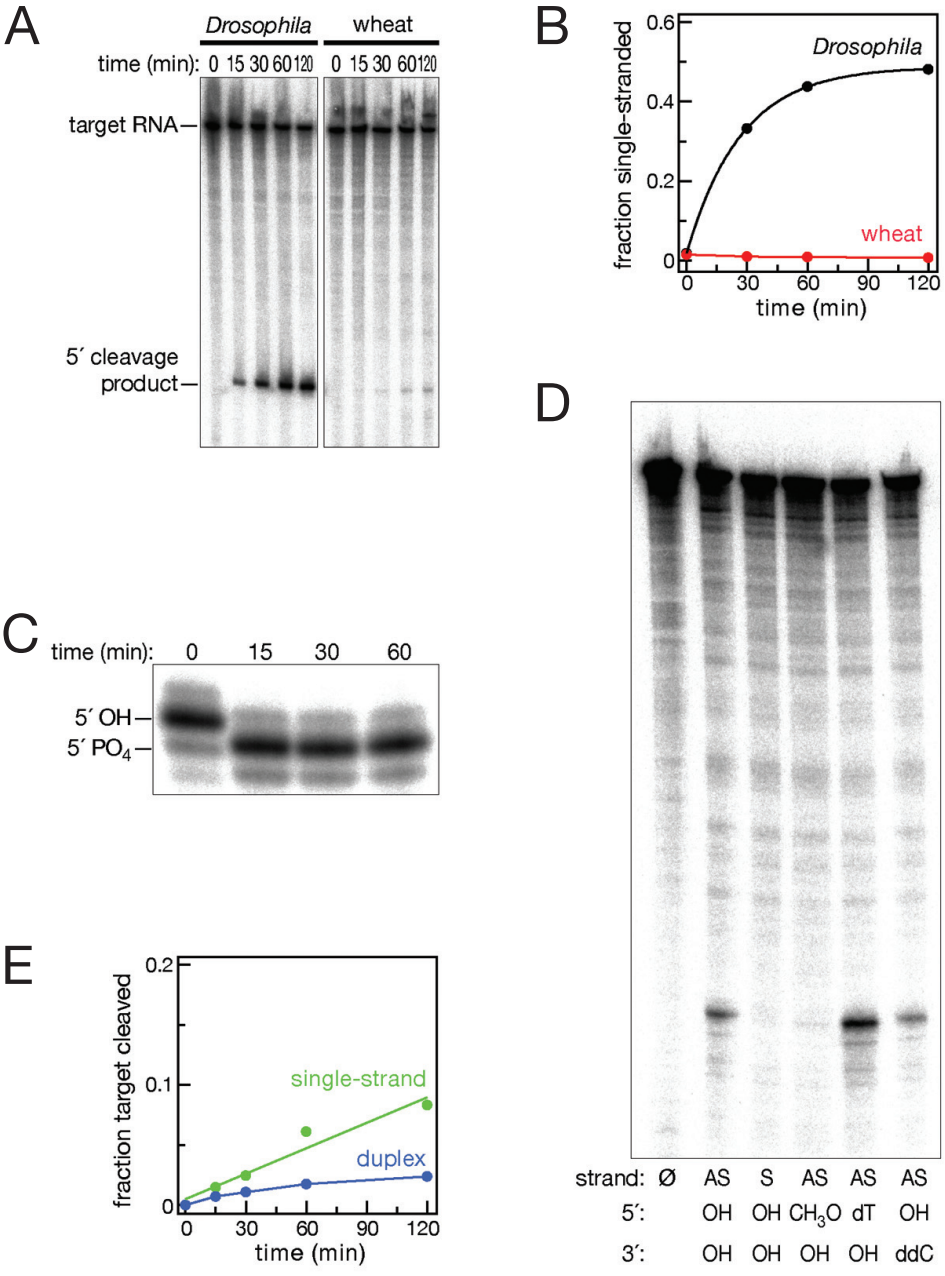

A
Drosophila
wheat
time (min): 0 15 30 60 120 0 15 30 60 120
target RNA
5′ cleavage product
B
fraction single-stranded
0.6
0.4
0.2
0
Drosophila
wheat
0 30 60 90 120
time (min)
C
time (min): 0 15 30 60
5′ OH
5′ PO4
D
strand: Ø AS S AS AS AS
5′: OH OH CH3O dT OH
3′: OH OH OH OH ddC
E
fraction target cleaved
0.2
0.1
0
single-strand
duplex
0 30 60 90 120
time (min)

Figure Legend II-1. Single-stranded but not double-stranded siRNA triggers RNAi in wheat germ extract. (**A**) Double-stranded siRNAs direct target cleavage in *Drosophila* embryo lysate (left), but triggered only low levels of target cleavage in wheat germ extract (right). (**B**) In the presence of ATP, *Drosophila* embryo lysate, but not wheat germ extract, unwound an siRNA duplex of which the guide strand was 5´-^{32}P-radiolabeled. (**C**) A 3´-^{32}P-radiolabeled siRNA duplex was rapidly phosphorylated upon incubation in wheat germ extract. (**D**) Single strand siRNAs containing 5´ and 3´ hydroxy (OH) termini directed efficient target cleavage in wheat germ extract. The siRNA was rapidly 5´ phosphorylated in the wheat germ extract, as shown in (C). A 5´ methoxy (CH_3O) modification, which blocks 5´ phosphorylation, inhibited target cleavage in wheat germ lysate. In order to introduce the 5´ methoxy modification, the first nucleotide of the siRNA was changed from riboU to deoxyT. This change, in the absence of the methoxy modification, did not inhibit target cleavage, as shown by the 5´ dT siRNA. A 2´,3´ dideoxy (ddC) modification of the 3´ end of the siRNA similarly had no significant effect on cleavage. The unmodified sense (S) siRNA triggered no target cleavage and is presented as a negative control. (**E**) Single-stranded (green) but not double-stranded (blue) siRNAs triggered efficient RNAi in wheat germ extracts. The appearance of the ~70 nt 5´ cleavage product was monitored over time.

target cleavage in wheat germ extracts. Single-stranded siRNAs functioned in wheat germ extract only when they contained a 5´ phosphate; the siRNAs did not cleave the target when the 5´ end was blocked by a methoxy group (Figure 1D), consistent with their guiding target cleavage as a component of RISC. A 5´ methoxy modification blocks RNAi in *Drosophila* embryo lysates and cultured human cells (Schwarz et al. 2002), because it blocks the assembly of the RLC (Tomari et al. 2004a). Thus, a 5´ phosphate is an essential feature of functional siRNAs in both plants and animals. In contrast, a 2´,3´ dideoxy-modified, single-stranded siRNA guided target cleavage in wheat germ extract, excluding a role for the siRNA as a primer of an RNA-dependent RNA polymerase acting to convert the target RNA into dsRNA that is subsequently destroyed by dicing. Over time, only a ~70 nt-product accumulated (Figure 1E), consistent with the single-stranded siRNA acting directly as a guide without involvement of an RdRP. In *Arabidopsis*, RdRP proteins are required for PTGS initiated by transgenes overproducing single-strand RNA (Dalmay et al. 2000; Mourrain et al. 2000), but not for target destruction initiated by dsRNA (Waterhouse et al. 1998).

Biochemical complementation of wheat germ RNAi

RNA silencing pathways are conserved between plants and animals. Therefore we asked if *Drosophila* proteins might complement the defect in siRNA unwinding in wheat germ extract. Wheat germ extract was supplemented with serial dilutions of *Drosophila* embryo lysate and incubated with double-stranded siRNA and an RNA target (Figures 2A and 2B). After incubation for 60 min, we assayed target cleavage (Figure

Figure II-2

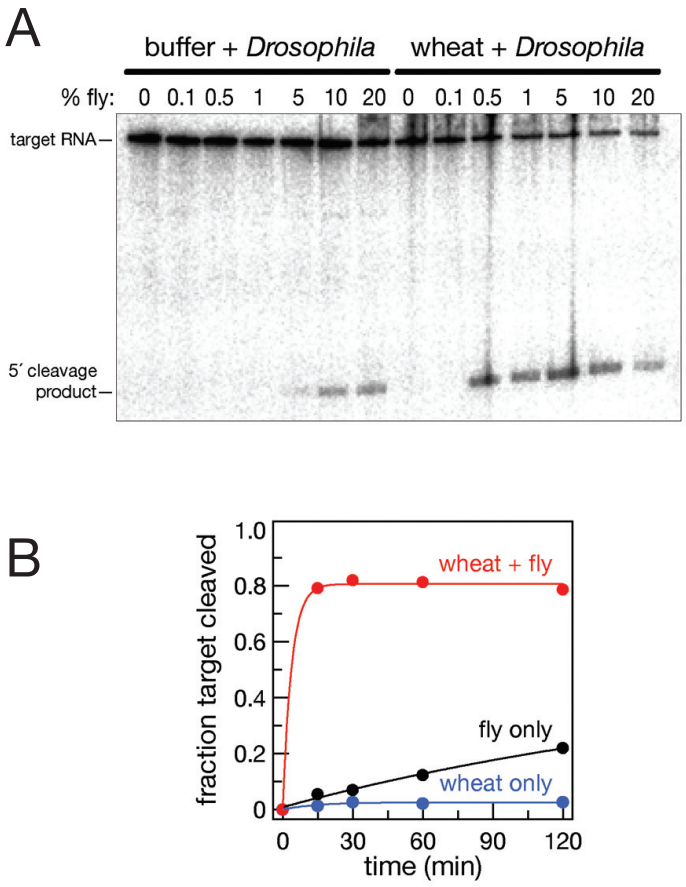

A
buffer + Drosophila
wheat + Drosophila
% fly: 0 0.1 0.5 1 5 10 20 0 0.1 0.5 1 5 10 20
target RNA
5′ cleavage product
B
fraction target cleaved
1.0
0.8
0.6
0.4
0.2
0
wheat + fly
fly only
wheat only
0 30 60 90 120
time (min)

Figure Legend II-2. *Drosophila* RNAi components complement the defect in wheat germ extract, allowing double-stranded siRNA to trigger target cleavage. (**A**) When wheat germ extract was supplemented with as little as 0.5 percent *Drosophila* embryo lysate, by volume, double-stranded siRNA directed target RNA cleavage. On its own, this amount of *Drosophila* embryo lysate was insufficient to trigger target cleavage, demonstrating that the *Drosophila* components complement the defect in wheat germ extract. (**B**) When the wheat germ was supplemented with *Drosophila* embryo lysate (red; 5 percent of the wheat germ reaction, by volume) nearly all the target RNA was converted to 5´ cleavage product by 15 min. By contrast, little or no target cleavage was observed for the wheat germ extract alone (blue) at 2 h. At that time, the diluted embryo lysate (black) had converted only ~20 percent of the target into 5´ cleavage product.

2A). Supplementing the wheat germ extract with as little as 1 part *Drosophila* embryo lysate per 200 activated the wheat germ extract for double-stranded siRNA-directed target cleavage. This amount of *Drosophila* embryo lysate on its own did not support RNAi, demonstrating that our assay measures biochemical complementation of wheat germ extract by *Drosophila* proteins. Thus, *Drosophila* embryo lysate complements wheat germ RNAi activity, even though the two organisms diverged during evolution over 200 million years ago (Hedges 2002).

Mutant Drosophila ovary lysates rescue the wheat defect

Since wild-type *Drosophila* embryo lysate rescued wheat germ, we asked if ovary lysates from *Drosophila* mutants that are defective in RNAi could rescue wheat germ RNAi (Tomari et al. 2004a; Tomari et al. 2004b). Wild-type *Drosophila* ovary complemented the wheat germ extract for RNAi (Figure 3A). Lysate from *dicer-2*, *r2d2*, and *armitage* mutant ovaries are all defective in loading RISC with siRNA. We therefore determined if ovary lysate from these mutants can complement wheat germ extract, allowing it to support target cleavage triggered by double-stranded siRNA. We supplemented wheat germ extract with ovary lysate from mutant flies defective for RNAi in vivo and in vitro: *dcr-2*^{L811fsX} and *dcr-2*^{G31R}, *r2d2*, and *armi*$^{72.1}$. *armi*$^{72.1}$ lysate rescued the wheat germ defect (Figure 3B), whereas ovary lysate from *r2d2* (Figure 3C) and *dcr2*^{L811fsX} (Figure 3D) flies did not complement wheat germ RNAi. Dicer-2 and R2D2 are required for siRNA-mediated RNAi (Liu et al. 2003; Lee et al. 2004; Pham et al. 2004), because they are core components of the RLC (Tomari et al. 2004a), which

initiates siRNA unwinding (Tomari et al. 2004b). In contrast, *dcr-2*^{G31R} mutant lysate rescued the defect in wheat germ at a concentration insufficient to support RNAi on its own (Figure 3E). The *dcr-2*^{G31R} mutation prevents *Drosophila* Dcr-2 protein from dicing long dsRNA into siRNAs, but preserves its function in unwinding siRNA duplexes and loading one of the two siRNA strands into RISC (Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004b). Moreover, recombinant Dicer2/R2D2 heterodimer, which can dice long dsRNA in vitro, did not rescue wheat germ RNAi (Figure 3F). Together these data suggest that multiple RLC components are missing from wheat germ extract and that these components are essential for the transfer of one of the siRNA strands from the double-stranded siRNA into the single-strand-containing, active RISC.

Next, we tested if *Drosophila* Argonaute2 (Ago2) is required to complement wheat germ extract. Ago2 is required in flies for siRNA unwinding (Okamura et al. 2004), because the RLC will not initiate siRNA unwinding in the absence of Ago2 (Tomari et al. 2004b). Although Ago2 is a core component of RISC and is the endonuclease that "slices" the target RNA (Liu et al. 2004; Meister et al. 2004; Parker et al. 2004; Song et al. 2004), it is not known if its endonuclease activity is required for its role in RISC assembly. We prepared ovary lysates from *ago2*^{16608} flies, a strong *ago2* allele. Intriguingly, *ago2*^{16608} lysates, unlike those from *armi*$^{72.1}$ mutant flies, did not rescue the wheat germ RNAi defect (Figure 3G), suggesting that wheat germ extract requires the function of the RLC and that the wheat germ extract lacks the RISC assembly machinery.

Figure II-3

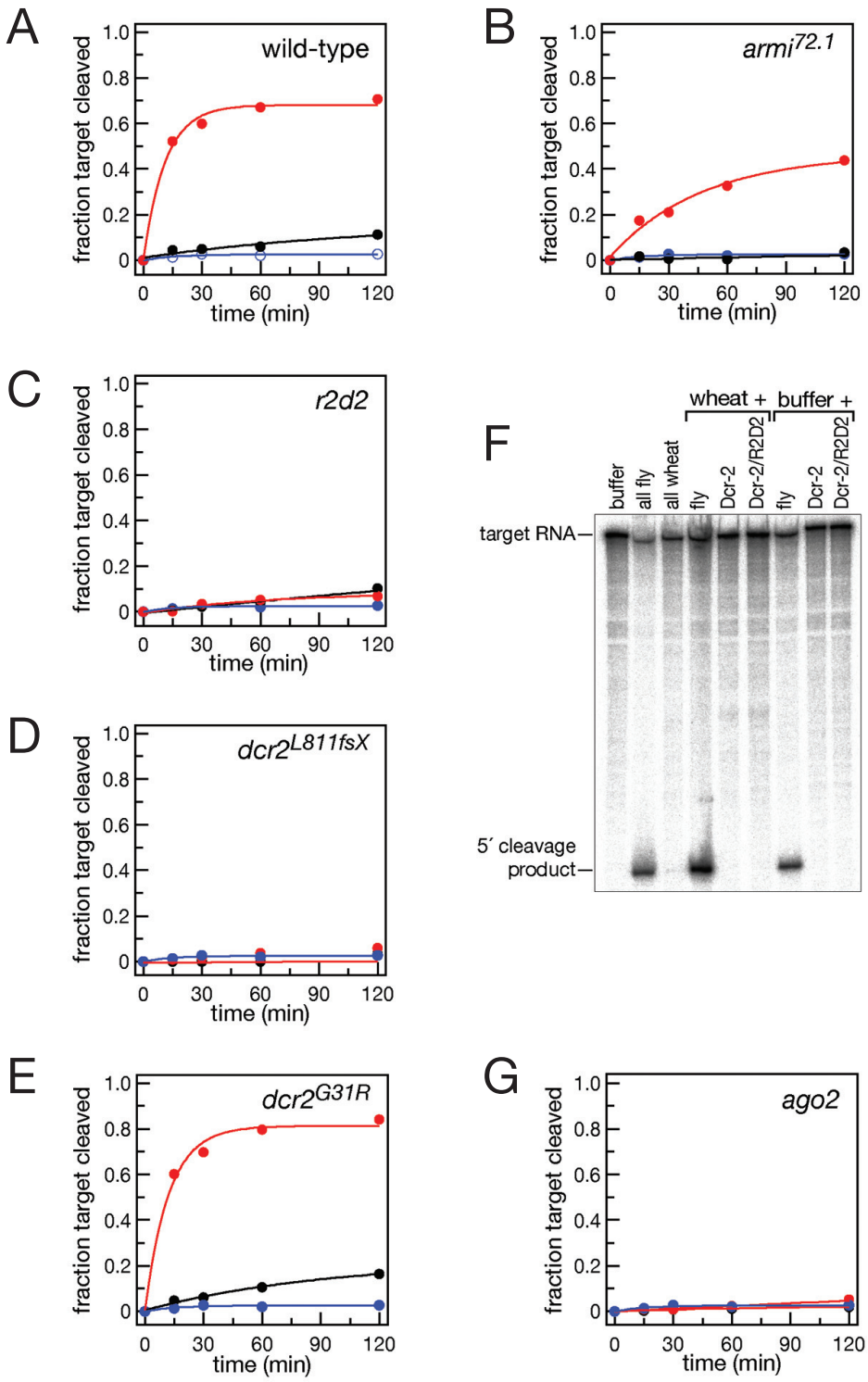

A
wild-type
B
armi72.1
C
r2d2
D
dcr2L811fsX
E
dcr2G31R
G
ago2
fraction target cleaved
time (min)
F
wheat +
buffer +
buffer
all fly
all wheat
fly
Dcr-2
Dcr-2/R2D2
fly
Dcr-2
Dcr-2/R2D2
target RNA
5′ cleavage product

Figure Legend II-3. Biochemical complementation of target cleavage (RNAi) directed by double-stranded siRNA in wheat germ extract by *Drosophila* ovary lysates. Panels **A-E** and **G** each show a time course of target RNA cleavage directed by a double-stranded siRNA in wheat germ extract alone (blue), *Drosophila* ovary lysate alone (black), and wheat germ extract supplemented with *Drosophila* ovary lysate (red). Wild-type (**A**) and *armitage* (*armi*$^{72.1}$) (**B**) ovary lysate complemented the wheat germ lysate. In contrast, ovary lysate from *dcr-2*^{l811fsX} (**C**) or *r2d2* (**D**) did not. Ovary lysate from the *dcr-2*^{G31R} allele (**E**), which is defective for dicing long dsRNA into siRNAs, but not for unwinding siRNA duplexes, complemented wheat germ extract. (**F**) Recombinant Dcr-2 protein or recombinant Dcr-2/R2D2 heterodimer were not sufficient to rescue wheat germ, when used at a physiologically relevant concentration. (**G**) Ovary lysates from flies mutant for *ago2* did not rescue the RNAi in wheat germ extract.

Conclusions

Here, we show that single-stranded siRNAs direct target RNA cleavage in wheat germ extracts. Like RNAi in animals, a 5´ phosphate is required for this siRNA function. Reconstitution of immuno-purified human Argonaute2 similarly requires a 5´ phosphate (Liu et al. 2004), and structural and kinetic studies argue that Argonaute proteins in general contain a pocket that binds the siRNA 5´ end, perhaps via phosphate interactions (Haley and Zamore 2004; Parker et al. 2004). Our data are consistent with this phosphate-binding pocket also being conserved in plant Argonaute proteins.

Although single-stranded siRNAs can trigger RNAi in wheat germ extract, these extracts cannot be programmed with double-stranded siRNA. Our data suggest that siRNA duplexes do not enter the RNAi pathway in wheat germ extract because they are not unwound. Biochemical complementation experiments with wild-type and mutant *Drosophila* ovary lysates show that wheat germ extract lacks multiple components of the RISC-loading machinery. We do not know if this reflects the absence of these components from the extract or if they are not present in intact wheat embryos. In contrast, wheat embryos clearly contain miRNA-programmed RISCs, since many functional miRNAs are present in wheat germ extract (G. Tang and PDZ, unpublished). Wheat embryos may contain a functional RISC-assembly machinery dedicated strictly to the production of miRNA-containing RISC, but may lack the comparable assembly machinery for siRNAs. Alternatively, RISC may be loaded with miRNAs during wheat germ cell development, and mature wheat embryos may lack some or all RISC assembly components.

Ovary lysates lacking the *Drosophila* protein *armitage*, which is required for RISC assembly and RNAi in vitro and in vivo (Tomari et al. 2004a) (Cook et al. 2004) complement wheat germ extracts for RNAi triggered by siRNA duplexes. The Armitage protein is the animal homolog of the plant protein SDE3, which may be present in plant cell extracts. In contrast, *ago2*^{16608}, *r2d2*, and *dicer2*^{L811fsX} mutant lysates, all of which are defective in siRNA unwinding and RISC assembly, do not complement wheat germ extract. Thus, at least three different *Drosophila* RISC assembly proteins are required to rescue the defect in wheat germ extract.

Materials and Methods

General Methods

Wheat germ extract preparation (Tang et al. 2003), target cleavage assays (Haley et al. 2003), RNAi triggered with single-stranded siRNAs (Schwarz et al. 2002), siRNA phosphorylation and unwinding assays (Nykänen et al. 2001), and *Drosophila* ovary lysate preparation (Tomari et al. 2004a) were as described previously.

siRNAs

siRNAs were prepared by standard synthesis (Dharmacon Research). Antisense siRNAs targeting firefly luciferase mRNA were: 5´-HO-UCG AAG UAU UCC GCG UAC GUG-3´(5´ OH, riboU); 5´-CH_3O-dTCG AAG UAU UCC GCG UAC GUG-3´ (5´ CH_3O, dT); 5´-HO-UCG AAG UAU UCC GCG UAC GUddC (2´,3´dideoxyC). siRNAs bearing a guide strand with the sequence of the animal miRNA *let-7*: 5´-HO-UGA GGU

AGU AGG UUG UAU AGU-3´. Sense strands used were 5´-HO-CGU ACG CGG AAU ACU UCG AAA-3´ for *Pp*-luc and 5´-HO-GCU ACA ACC UAC UAC CUC CUU-3´ for the *let-7* siRNA. Sense and antisense strands were annealed as described (Elbashir et al. 2001a). siRNAs were labeled using polynucleotide kinase (New England Biolabs) and γ-^{32}P-ATP (NEN) or poly(A) polymerase (Life Technologies) and α-^{32}P-cordycepin-5´-triphosphate (NEN) as described (Haley et al. 2003).

CHAPTER III: Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2 Containing RNAi Enzyme Complexes

Disclaimer: The following chapter was a collaborative effort. All the experiments for this publication were done by the author with the following exceptions: Figures 2B and 6 were a collaboration between Yukihide Tomari and myself; Figures 7 and 8 as well as Supplemental Figure 6 was a collaborative effort with Chanseok Shin and David Bartel.

Summary

In the *Drosophila* and mammalian RNA interference pathways, siRNAs direct the protein Argonaute2 (Ago2) to cleave corresponding mRNA targets, silencing their expression. Ago2 is the catalytic component of the RNAi enzyme complex, RISC. For each siRNA duplex, only one strand, the guide, is assembled into the active RISC; the other strand, the passenger, is destroyed. An ATP-dependent helicase has been proposed first to separate the two siRNA strands, then the resulting single-stranded guide is thought to bind Ago2. Here, we show that Ago2 instead directly receives the double-stranded siRNA from the RISC assembly machinery. Ago2 then cleaves the siRNA passenger strand, thereby liberating the single-stranded guide. For siRNAs, virtually all RISC is assembled through this cleavage-assisted mechanism. In contrast, passenger-strand cleavage is not important for the incorporation of miRNAs that derive from mismatched duplexes.

Introduction

RNA interference (RNAi) begins with the production of ~21 nt, double-stranded RNAs called small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001b; Bernstein et al., 2001; Elbashir et al., 2001a). siRNAs must be dissociated into their component single strands in order to act as guides for the protein complexes that repress gene expression (Nykänen et al., 2001; Martinez et al., 2002). In humans and flies, the best-studied RNAi protein complex is the RNA-induced silencing complex (RISC), in which the siRNA directs the protein Argonaute2 (Ago2) to the RNA target (Hammond et al., 2001). Ago2 is the sole siRNA-guided Argonaute protein able to act as an RNA-guided RNA endonuclease in both flies and humans (Okamura et al., 2004; Meister et al., 2004b; Rand et al., 2004; Liu et al., 2004; Rivas et al., 2005). Ago2, a Mg^{2+}-dependent endonuclease, cleaves a single phosphodiester bond in the target RNA, triggering its destruction (Rand et al., 2004; Liu et al., 2004; Song et al., 2004; Rivas et al., 2005). In *Drosophila melanogaster*, dissociation of the two siRNA strands requires the Dicer-2 (Dcr-2)/R2D2 protein heterodimer, as well as Ago2 itself (Liu et al., 2003; Lee et al., 2004; Pham et al., 2004; Okamura et al., 2004). Dcr-2 and R2D2 act in the RISC-loading complex (RLC) to sense the relative local stabilities of the 5´ ends of each siRNA strand, preferentially loading into Ago2 the siRNA strand less tightly base paired at its 5´ end (Tomari et al., 2004b). In *Drosophila* embryo lysate lacking Ago2, siRNAs remain double-stranded (Okamura et al., 2004) and stably bound to Dcr-2 and R2D2 (Tomari et al., 2004b), suggesting that siRNA strand separation is initiated only after Ago2 interacts with the siRNA bound to

Dcr-2 and R2D2.

Previous models have proposed that an ATP-dependent helicase first separates the siRNA duplex, and then the resulting single-stranded guide binds Ago2 (Zamore et al., 2000; Nykänen et al., 2001; Bartel, 2004; Meister and Tuschl, 2004; Sontheimer, 2005; Tomari and Zamore, 2005). Here, we present experimental data supporting an alternative model. Our data indicate that the RLC loads an siRNA duplex, not an siRNA single strand, into Ago2. The orientation of Ago2 on the double-stranded siRNA, that is, which 5´ phosphate is lodged in the phosphate-binding pocket of the Ago2 Piwi domain, would be determined by the RISC-loading complex. Once bound to the siRNA duplex, Ago2 cleaves the passenger strand, triggering its dissociation from the complex and the concomitant maturation of the active RISC. Passenger-strand cleavage is not obligatory, but is the normal mechanism for loading siRNA into Ago2. For standard siRNAs, passenger-strand cleavage follows rapidly after Ago2 binds the double-stranded siRNA, but when cleavage is blocked by chemical modification or by mismatches between the siRNA guide and passenger strands, a slower 'back-up' pathway dissociates and destroys the passenger strand, liberating mature RISC. This bypass pathway explains the loading of miRNAs into RISC and the loading of silencing RNAs into complexes that are not capable of Ago-mediated cleavage.

Results

Passenger, but not Guide, Strand Cleavage Accompanies RISC Assembly

To test this alternative model, we asked if the passenger strand is cleaved during

RISC assembly. Human and *Drosophila* Ago2 cleave their target RNAs at the phosphodiester bond that lies across from nucleotides 10 and 11 of the guide strand (Elbashir et al., 2001c; Elbashir et al., 2001b; Martinez et al., 2002; Schwarz et al., 2002; Haley and Zamore, 2004; Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). The equivalent phosphodiester bond lies between nucleotides 9 and 10 of the passenger strand. Cleavage of the passenger strand, directed by the siRNA guide strand and catalyzed by Ago2, is therefore predicted to yield a 9-nt 5′ cleavage product. To determine if the siRNA passenger strand is cleaved during RISC assembly, we 5′-^{32}P-radiolabeled each strand of a highly asymmetric siRNA targeting the human sod1 mRNA (Figure III-1A). This siRNA loads one strand (red) to the near exclusion of the other (blue), because it contains an unpaired nucleotide at the 5′ end of the guide (Schwarz et al., 2003). Upon incubation in *Drosophila* embryo lysate, the radiolabeled passenger strand was converted to a 9 nt, 5′-radiolabeled species whose accumulation peaked at 5 min, then disappeared (Figure III-1A). In contrast, no 9-nt product was detected for the guide strand. The 9-nt passenger-strand cleavage fragment was also produced when this siRNA was incubated in S2 cell lysate that supports in vitro Ago2 RISC assembly and RNAi (Supplemental Figure III-1), showing that the phenomenon was not restricted to syncitial blastoderm embryos.

Next, we 5′-radiolabeled each strand of a second highly asymmetric siRNA in which the guide (red) had the sequence of the microRNA *let-7*. Again, when the siRNA was incubated in an in vitro RNAi reaction, we detected a 9-nt, 5′-radiolabeled product for the passenger strand (blue), but not the guide (Figure III-1B). We also examined an

Figure III-1

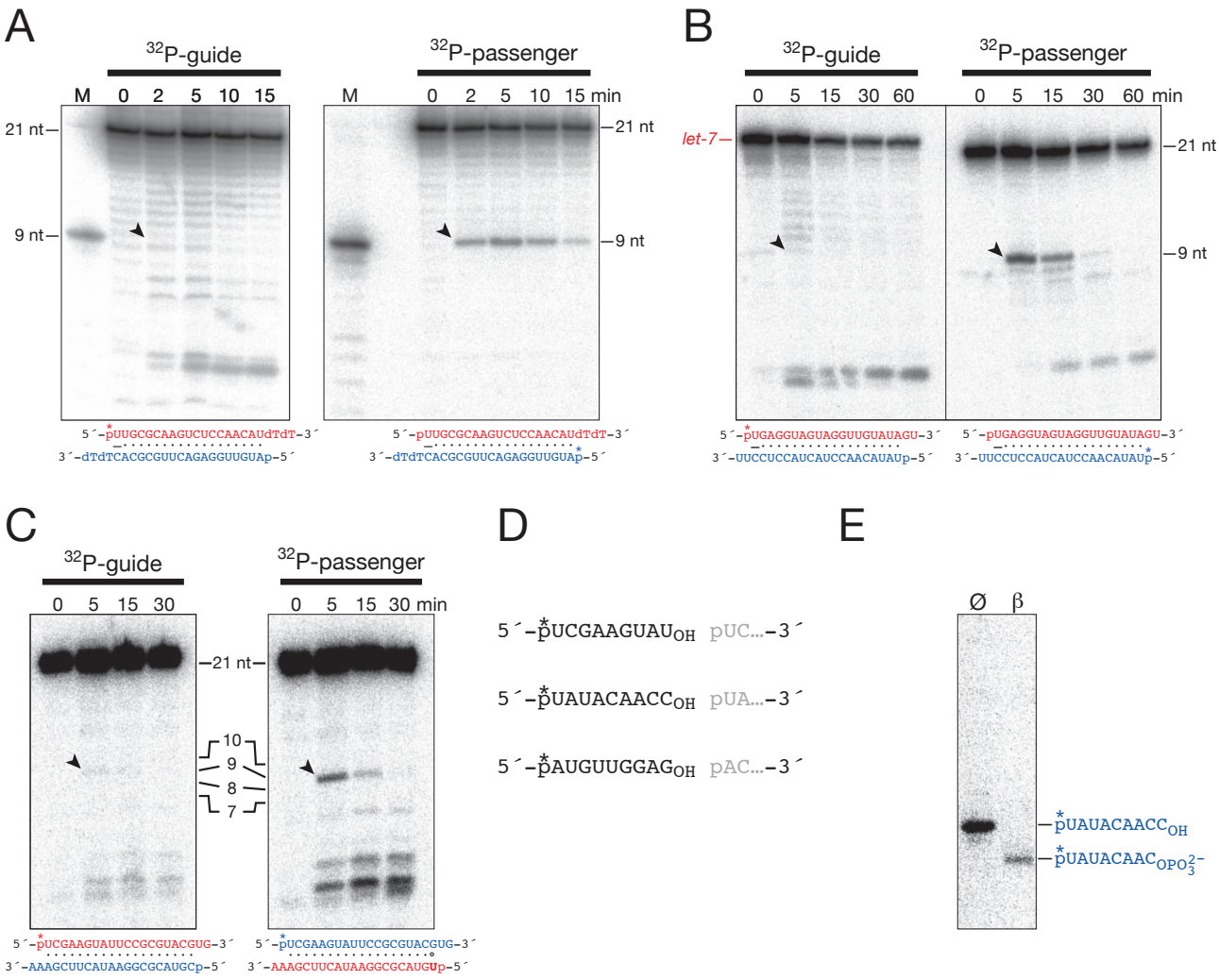

A
32P-guide
M 0 2 5 10 15
21 nt
9 nt
32P-passenger
M 0 2 5 10 15 min
21 nt
9 nt
5´-pUUGCGCAAGUCUCCAACAUdTdT-3´
3´-dTdTCACGCGUUCAGAGGUUGUAp-5´
5´-pUUGCGCAAGUCUCCAACAUdTdT-3´
3´-dTdTCACGCGUUCAGAGGUUGUAp-5´
B
32P-guide
0 5 15 30 60
let-7
32P-passenger
0 5 15 30 60 min
21 nt
9 nt
5´-pUGAGGUAGUAGGUUGUAUAGU-3´
3´-UUCCUCCAUCAUCCAACAUAUp-5´
5´-pUGAGGUAGUAGGUUGUAUAGU-3´
3´-UUCCUCCAUCAUCCAACAUAUp-5´
C
32P-guide
0 5 15 30
32P-passenger
0 5 15 30 min
21 nt
10
9
8
7
5´-pUCGAAGUAUUCCGCGUACGUG-3´
3´-AAAGCUUCAUAAGGCGCAUGCp-5´
5´-pUCGAAGUAUUCCGCGUACGUG-3´
3´-AAAGCUUCAUAAGGCGCAUGUp-5´
D
5´-pUCGAAGUAUOH pUC...-3´
5´-pUAUACAACCOH pUA...-3´
5´-pAUGUUGGAGOH pAC...-3´
E
Ø β
pUAUACAACCOH
pUAUACAACOPO32-

Figure Legend III-1. The siRNA passenger strand, but not the guide, is cleaved between nucleotides 9 and 10 during RISC assembly in *Drosophila* embryo lysate. (A) An siRNA targeting human superoxide dismutase 1 (sod1), 5´-^{32}P-radiolabeled on either the guide or the passenger strand was incubated in an in vitro RNAi reaction. For the passenger strand, a 9-nt 5´ cleavage product accumulated early in the reaction, then disappeared. (B) A similar experiment, using an siRNA in which the guide strand had the sequence of the *let-7* microRNA and the passenger strand was antisense to *let-7*. Again, a 9-nt 5´cleavage product was detected only for the passenger. (C) Two variants of an siRNA targeting firefly luciferase were incubated in an in vitro RNAi reaction. For both, the antisense strand was ^{32}P-radiolabeled. When the radiolabeled, antisense strand served as passenger, a 9-nt 5´ cleavage product was observed, but not when the antisense strand acted as the guide. (D) The three different passenger cleavage site sequences suggest that cleavage is not sequence specific. (E) The 3´ termini of the purified *let-7* siRNA passenger-strand cleavage product were mapped by reaction with sodium periodate followed by β-elimination; the 5´ passenger-strand cleavage product bears 2´,3´ hydroxy termini, just as do the products of Ago2-mediated target RNA cleavage. Ø, gel isolated 9 nt passenger-strand cleavage product; β, the purified passenger-strand cleavage product reacted with sodium periodate followed by β-elimination.

siRNA targeting the firefly luciferase mRNA, 5´-^{32}P-radiolabeled on its anti-sense strand (red; Figure III-1C). When this siRNA is fully base-paired, the local thermodynamic difference between the two 5´ ends favors assembly of the anti-sense strand into RISC (Schwarz et al., 2003), making the radiolabeled strand the guide. Changing a C to a U at position 19 of the sense strand (blue) inverts the asymmetry of the siRNA, converting the radiolabeled strand into the passenger. When incubated in the RNAi reaction, only the siRNA in which the radiolabeled strand served as passenger produced a 9 nt, 5´ product. These data indicate that cleavage is restricted to the passenger strand of the siRNA duplex.

The three siRNAs in Figure III-1 include examples in which the nucleotide 5´ to the cleavage site is a purine or a pyrimidine and in which the nucleotide 3´ to the cleavage site is a purine or a pyrimidine (Figure III-1D), suggesting that passenger-strand cleavage does not depend on siRNA sequence. Treatment of the purified 9-nt cleavage product with sodium periodate followed by β-elimination generated an 8 nt RNA bearing a 3´ phosphate, demonstrating that the 9-nt passenger-strand product contains a 2´,3´ hydroxy terminus (Figure III-1E). A 3´ hydroxy terminus is consistent with passenger-strand cleavage by Ago2, which cleaves target RNAs to yield 5´ cleavage products bearing a 3´ hydroxyl group (Martinez and Tuschl, 2004; Schwarz et al., 2004), but not by the RISC component Tudor-SN, which is a member of a nuclease family that leaves a 3´ phosphate on its products (Caudy et al., 2003).

Passenger-strand cleavage Requires Ago2 and RISC Assembly Factors

Consistent with the view that Ago2 cleaves the passenger strand, the 9-nt cleavage product was not produced when an siRNA, 5´ radiolabeled on the passenger strand, was incubated in ovary lysate from *ago2*^{414} mutant flies (Figure III-2A), which contain little (Deshpande et al., 2005) or no (Okamura et al., 2004) detectable Ago2 protein. These data also indicate that Dcr-2, which, like Ago2 produces 5´ cleavage products bearing 3´ hydroxy termini, is not the nuclease that cleaves the passenger strand, because *ago2*^{414} mutants contain normal levels of Dcr-2 protein (Tomari et al., 2004b), and Dcr-2 needs no protein cofactors to act as an endonuclease (Liu et al., 2003). Our model postulates that passenger-strand cleavage occurs as a normal step in Ago2 RISC loading and not as a consequence of association of the siRNA with Ago2 through a mechanism independent from the RISC-loading complex (RLC). Passenger strand cleavage should therefore require the core components of the RLC, Dcr-2 and R2D2 (Lee et al., 2004; Pham et al., 2004). Indeed, both Dcr-2 and R2D2 were required for cleavage of the passenger strand, in that ovary lysates prepared from *dcr2*^{L811fsX} and *r2d2* mutant flies do not cleave the passenger strand (Figure 2B).

The Passenger Strand is Cleaved Before the siRNA Strands Dissociate

We can envision two distinct mechanisms in which an siRNA passenger strand might act as a substrate for Ago2 cleavage. In the first, RISC assembly would proceed through an siRNA unwinding step, the single-stranded guide, freed from the passenger, would be assembled into RISC, and this mature RISC would then bind and cut the single stranded,

Figure III-2

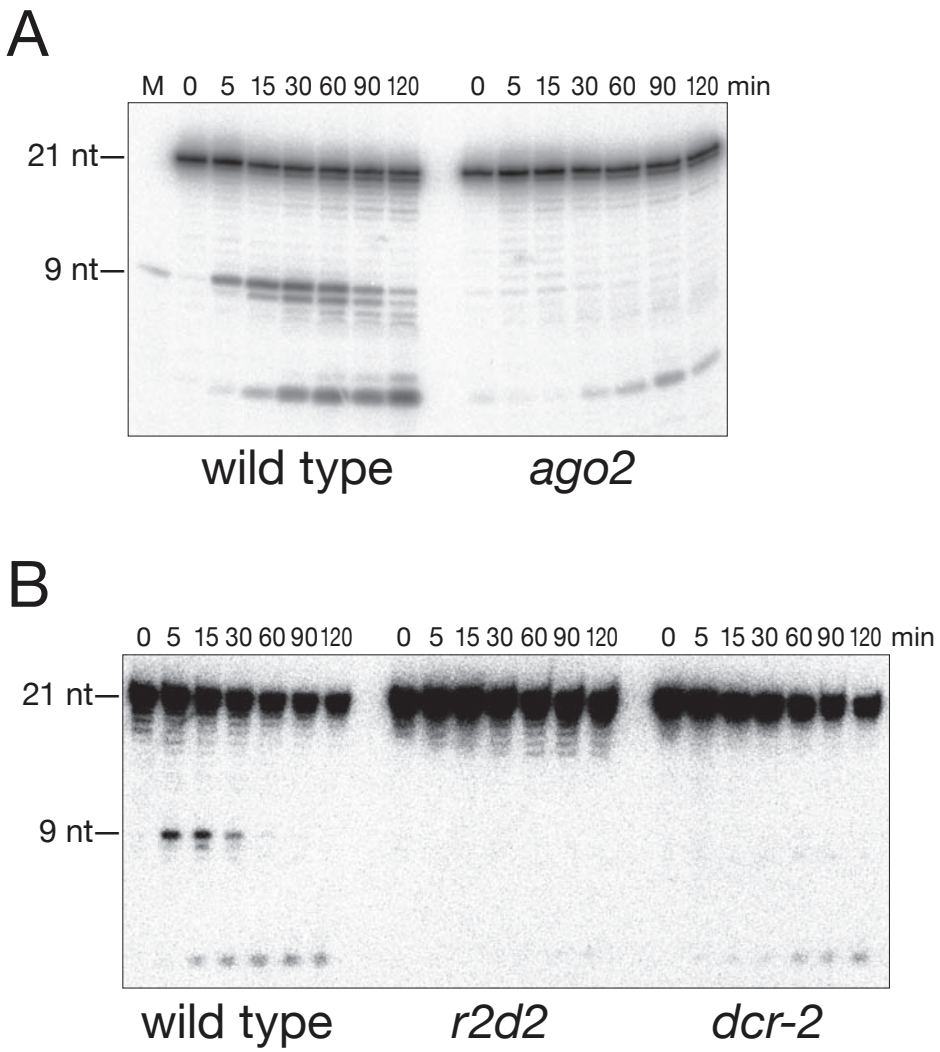

A
M 0 5 15 30 60 90 120 0 5 15 30 60 90 120 min
21 nt—
9 nt—
wild type
ago2
B
0 5 15 30 60 90 120 0 5 15 30 60 90 120 0 5 15 30 60 90 120 min
21 nt—
9 nt—
wild type
r2d2
dcr-2

Figure Legend III-2. Passenger-strand cleavage requires the RISC assembly machinery. (A) Ago2 is required for passenger-strand cleavage. *ago2*^{414} mutant ovary lysates produced little or no passenger-strand cleavage product compared to a wild-type ovary lysate control. (B) Ovary lysates prepared from *dcr-2*^{L811fsX} and *r2d2* mutant females do not cleave the passenger strand. These mutants cannot assemble the RISC-loading complex (RLC).

full-length passenger liberated by the RISC assembly process. In this model, passenger-strand cleavage is a consequence of RISC assembly, but not a step in the RISC assembly pathway. Because this *trans* mode of passenger-strand cleavage requires the function of mature RISC, it should be blocked by an antisense oligonucleotide that binds siRNA-programmed Ago2. In contrast, passenger-strand cleavage could occur in *cis* before the passenger ever dissociates from the guide strand, as envisioned in our alternative model for RISC assembly. Here, the double-stranded siRNA would be loaded into Ago2 RISC with a defined orientation, Ago2 would cut the passenger, and then the cleaved passenger strand fragments would be liberated together with mature RISC programmed with single-stranded guide siRNA. In this model, an antisense inhibitor of RISC should have no effect on passenger-strand cleavage, although it would block the subsequent ability of RISC to cleave a bona fide target mRNA in *trans*.

We used the experimental scheme diagrammed in Figure III-3A to distinguish between these two possibilities. RISC was programmed by combining *Drosophila* embryo lysate, a double-stranded siRNA 5´-^{32}P-radiolabeled on the passenger strand, and a 29-nt long, 2´-*O*-methyl oligonucleotide in a standard in vitro RNAi reaction. The 2´-*O*-methyl oligonucleotide contained a central 21-nt region complementary to the guide strand of the siRNA, so that it could bind and block the function of mature RISC. Such 2´-*O*-methyl oligonucleotides act as stoichiometric inhibitors of RISC function in vitro and in vivo (Hutvágner et al., 2004; Meister et al., 2004a; Leaman et al., 2005). A 2´-*O*-methyl oligonucleotide with a sequence unrelated to the siRNA was used as a control. We monitored the production of cleaved passenger strand for both the experimental and

Figure III-3

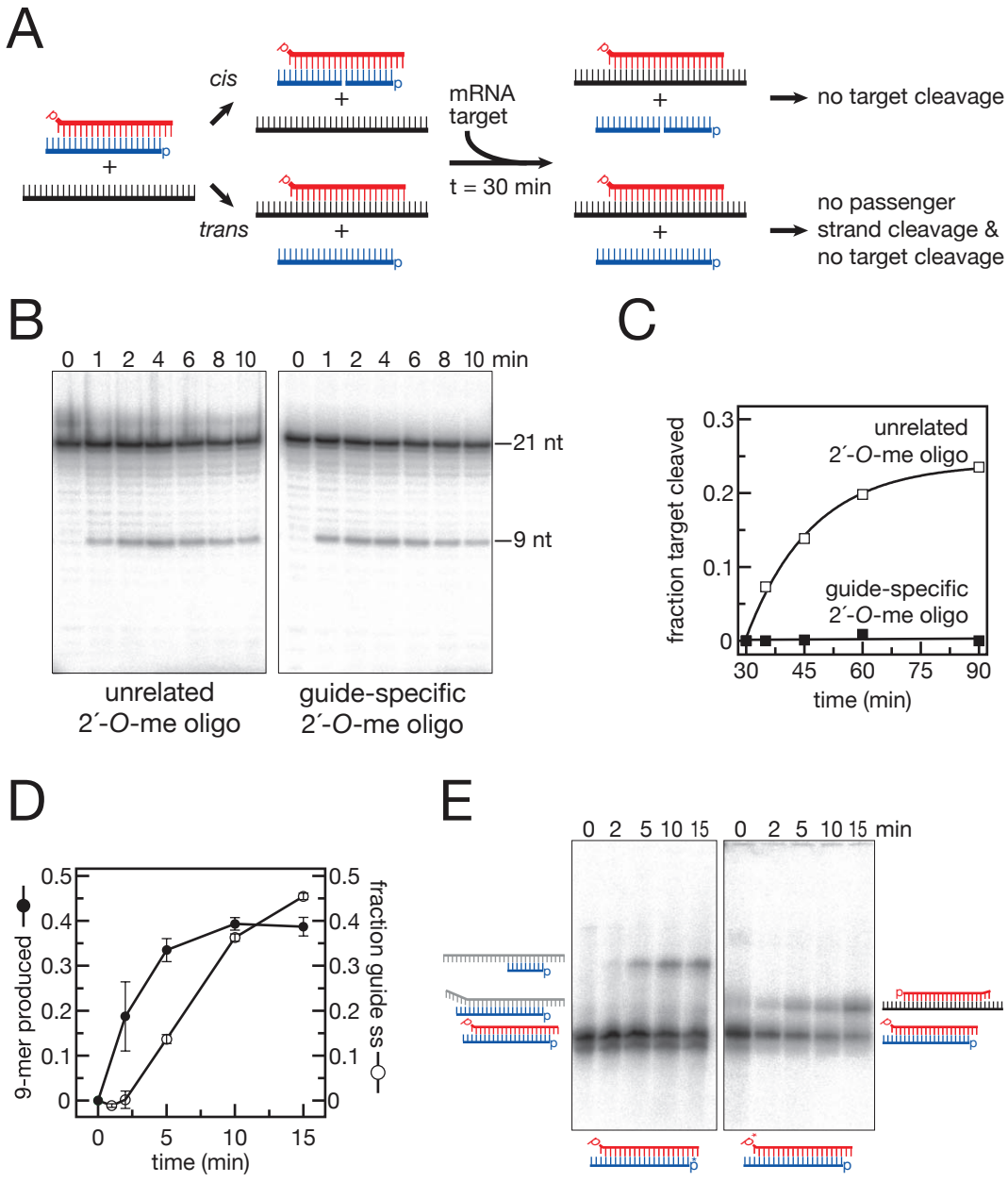

A
cis
trans
mRNA target
t = 30 min
no target cleavage
no passenger strand cleavage & no target cleavage
B
0 1 2 4 6 8 10 0 1 2 4 6 8 10 min
21 nt
9 nt
unrelated 2′-O-me oligo
guide-specific 2′-O-me oligo
C
fraction target cleaved
unrelated 2′-O-me oligo
guide-specific 2′-O-me oligo
time (min)
D
9-mer produced
fraction guide ss
time (min)
E
0 2 5 10 15 0 2 5 10 15 min

Figure Legend III-3. The passenger strand is cleaved before the two siRNA strands dissociate. (A) Experimental design. A 2´-*O*-methyl oligonucleotide (black) bearing the same sequence as the target of the guide strand (red) or an unrelated control 2´-*O*-methyl oligonucleotide was incubated with double-stranded siRNA in an in vitro RISC assembly reaction. Passenger-strand cleavage was monitored for the first 10 min. RISC assembly was continued until 30 min, when assembly was quenched, and target RNA added. Target cleavage was monitored for an additional 60 min, i.e., 90 min after initiating RISC assembly. The sequence of the siRNA appears in Figure III-1A. (B) Passenger-strand cleavage was unaffected by the guide-specific 2´-*O*-methyl oligonucleotide. (C) In contrast, the guide-specific 2´-*O*-methyl oligonucleotide, but not the control oligonucleotide, inactivated mature RISC. The data suggest that the passenger strand was cleaved in *cis*, before the two siRNA strands dissociated to liberate mature RISC. (D) The rate of passenger-strand cleavage and of the production of single-stranded guide strand—a measure of mature RISC production—were monitored for the siRNA used in (B) and (C). Each data point represents the average of three trials ± standard deviation. To prevent 9-mer degradation, a 100-fold excess of 2´-*O*-methyl oligonucleotide, relative to siRNA, was included in the reaction. (E) When RISC was assembled under standard in vitro conditions, full-length (21-nt) guide strand was readily trapped by a complementary 2´-*O*-methyl oligonucleotide. In contrast, we could not detect the production of full-length passenger strand when a passenger-complementary 2´-*O*-methyl oligonucleotide trap was included. Instead, we observed accumulation of the 9-nt 5´ passenger-strand cleavage product. A small amount of single-stranded full-length passenger strand, bound

to the 2´-O-methyl oligonucleotide, was detected at the beginning of the reaction and was, therefore, not a consequence of RISC assembly. The identity of the bands was assigned by their comigration with synthetic RNA:RNA or 2´-*O*-methyl oligonucleotide:RNA duplexes as diagrammed.

the control reactions. After 30 min, RISC assembly was quenched by treatment with *N*-ethylmaleimide (Nykänen et al., 2001), then a ^{32}P-radiolabeled target RNA was added to the reaction, and we monitored target cleavage, a measure of the concentration of active RISC. Neither 2´-*O*-methyl oligonucleotide had any detectable effect on passenger strand cleavage (Figure III-3B). Yet, in the same reaction, the 2´-*O*-methyl oligonucleotide complementary to the siRNA guide strand, but not the control oligonucleotide, completely blocked target cleavage (Figure III-3C). These data indicate that passenger strand cleavage occurs in *cis*, before the two siRNA strands separate.

In further support of the cis cleavage model, we found that passenger-strand cleavage of a standard, double-stranded siRNA preceded the production of single stranded guide strand, i.e., the production of mature RISC (Figure III-3D). In order to accurately measure the rate of production of cleaved passenger strand, the reaction included a 2´-*O*-methyl oligonucleotide trap complementary to the 5´-^{32}P-radiolabeled passenger strand. The trap oligo can bind single-stranded, cleaved passenger strand, helping to protect it from subsequent degradation (Supplemental Figure III-S2 and below). At each time point, cleaved passenger strand was resolved from full-length passenger strand by denaturing gel electrophoresis. Single-stranded guide production was measured in parallel by native gel analysis using the same siRNA duplex, except the siRNA was 5´-^{32}P-radiolabeled on the guide.

These two assays revealed that nearly one-fifth of the passenger strand was cleaved after 2 min incubation, a time when little or no single-stranded guide could be detected (Figure III-3D). This observation was counter to the *trans* model of passenger-

strand cleavage, in which single-stranded guide would need to form prior to 9-mer production, but it concurred with the *cis* model, in which single-stranded guide must appear after or concurrently with 9-mer production. The observed lag between 9-mer production and single-stranded guide formation suggests that passenger-strand fragments remain paired to the guide strand for several minutes after cleavage. Only after these fragments dissociate, is the guide strand liberated and available for detection as single stranded.

Control experiments (not shown) demonstrated that although the passenger strand-specific trap oligonucleotide significantly improved capture of the 9-nt species produced by passenger-strand cleavage, the 9-mer was eventually degraded even in the presence of the trap. Thus, our method underestimated the amount of 9-mer produced from the passenger strand and therefore likely underestimated the speed of this cleavage event relative to the production of mature RISC containing a single-stranded guide. We conclude that, within the limits of detection of our assay, one molecule of passenger strand was cleaved for every molecule of guide strand assembled into RISC. Supporting this view, we have been unable to detect the production of any full length, single-stranded passenger strand during RISC assembly (Figure III-3E). We incubated an siRNA, ^{32}P-radiolabeled on its passenger strand, in a standard RNAi reaction that contained a 29-nt, 2´-*O*-methyl oligonucleotide complementary in its central 21 nts to the passenger strand. At each time point, the samples were deproteinized at room temperature and then analyzed by native gel electrophoresis using a gel system containing 1.5 mM Mg^{2+} in both the gel and the running buffer. In parallel, the analysis was performed using the

same siRNA, but radiolabeled on the guide strand and using a 2´-*O*-methyl oligonucleotide complementary to the guide. We readily detected the production of single-stranded, full-length guide strand, but were unable to detect any full length, single stranded passenger strand, observing only the production of single stranded, 9 nt passenger-strand cleavage fragment.

A New Model For Assembly of siRNA into Ago2 RISC

Together, the results presented in Figures III-1, 2, and 3 suggest a revised model for the assembly of Ago2-containing RISC. We envision that the RLC first binds the double-stranded siRNA, its less tightly paired 5´ end perhaps lodged in the PAZ domain of Dcr-2 and the other end bound to the double-stranded RNA-binding domains of R2D2. The RLC then recruits Ago2 to the siRNA, exchanging R2D2 for binding of the Ago2 PAZ domain, followed by the loading of the 5´ phosphate of the siRNA guide strand into the phosphate-binding pocket of the Ago2, releasing Dcr-2. At this step (yellow box *1* in Figure III-4A), the intact, double-stranded siRNA is bound to Ago2. Cleavage of the passenger strand by Ago2 would rapidly follow, producing a double-stranded siRNA bearing a nicked passenger strand (yellow box *2* in Figure III-4A). We do not know how the passenger strand fragments are subsequently liberated from Ago2, but by analogy to the dissociation of target cleavage products, which is assisted by ATP (Haley and Zamore, 2004), we draw passenger strand fragment dissociation as potentially facilitated by ATP. Release of the passenger strand fragments would liberate mature RISC. This model predicts the existence of a new siRNA species in the Ago2 RISC assembly

Figure III-4

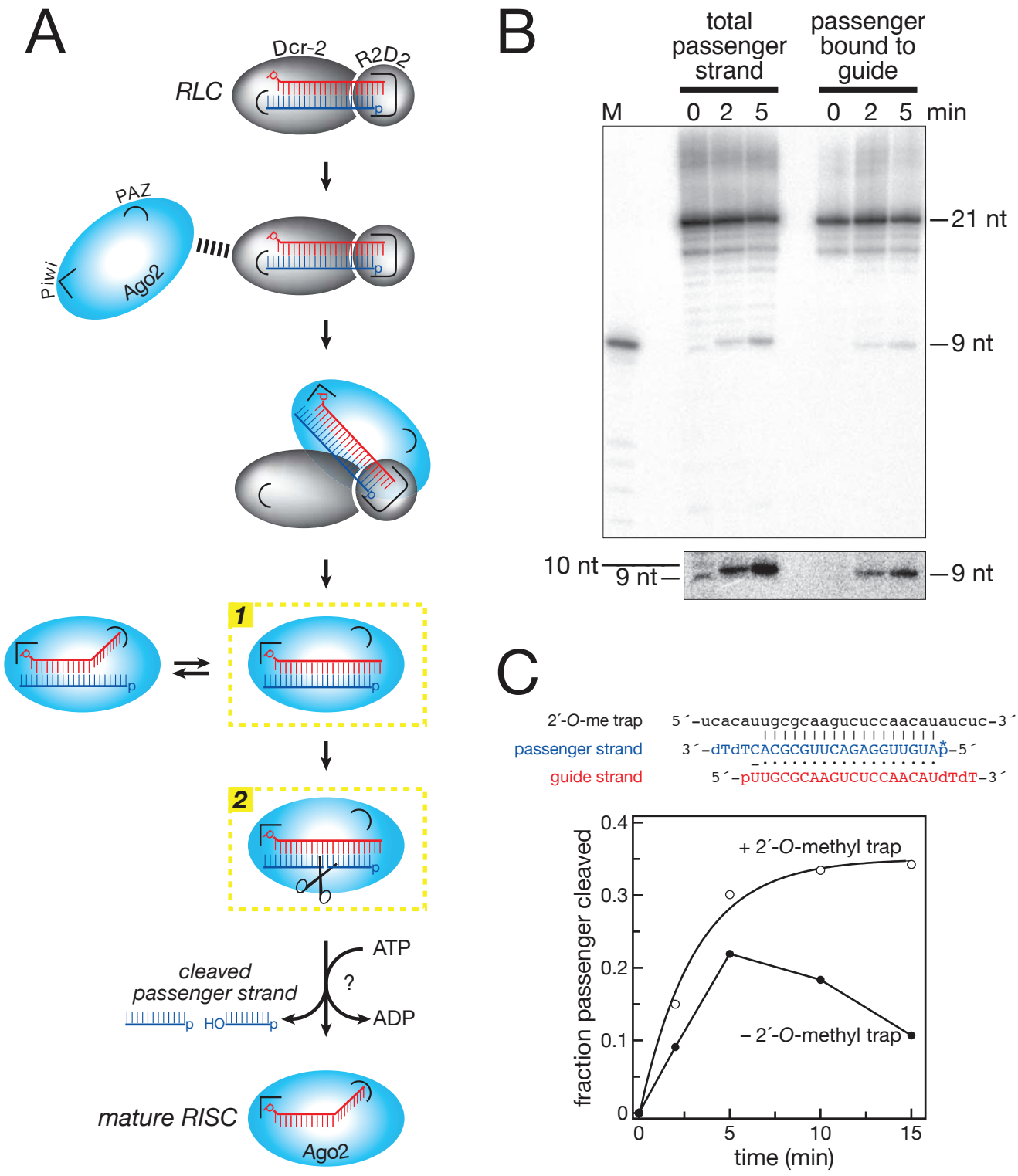

A
Dcr-2
R2D2
RLC
PAZ
Piwi
Ago2
1
2
cleaved
passenger strand
ATP
?
ADP
mature RISC
Ago2
B
total
passenger
strand
passenger
bound to
guide
M
0 2 5
0 2 5 min
21 nt
9 nt
10 nt
9 nt
9 nt
C
2′-O-me trap
passenger strand
guide strand
5′-ucacauugcgcaagucuccaacauaucuc-3′
3′-dTdTCACGCGUUCAGAGGUUGUAp-5′
5′-pUUGCGCAAGUCUCCAACAUdTdT-3′
+ 2′-O-methyl trap
– 2′-O-methyl trap
fraction passenger cleaved
0.4
0.3
0.2
0.1
0
0
5
10
15
time (min)

Figure Legend III-4. A revised model for RISC assembly. (A) Dcr-2 and R2D2 are envisioned to recruit Ago2 directly to the double-stranded siRNA. Ago2 exchanges first with Dcr-2, with whom it makes a protein-protein contact (dashed line) (Tahbaz et al., 2004), then with R2D2. Finally, Ago2 cleaves the passenger strand (blue), thereby liberating the guide (red) from the siRNA duplex and producing active RISC. Release of the passenger-strand cleavage products may be facilitated by an ATP-dependent cofactor, much as release of the products of target cleavage facilitated by ATP (Haley and Zamore, 2004). We have included conformational rearrangements in Ago2 proposed to be associated with target RNA cleavage (Tomari and Zamore, 2005). (B) Two-dimensional analysis of the double-stranded population provides for support for the model. Double stranded siRNA, 5´-^{32}P-radiolabled on the passenger strand, was incubated in a RISC assembly reaction in the presence of a 29-nt 2´-*O*-methyl oligonucleotide complementary to the passenger strand, then the trap-bound and free RNA species were separated by native gel electrophoresis as diagrammed in Supplemental Figure S2. The region of the gel corresponding to double-stranded siRNA was excised and its RNA constituents analyzed by denaturing electrophoresis. The double-stranded siRNA population comprised both species *1* and species *2* postulated by the model in (A). That is, a portion of the 'double-stranded' siRNA contains a cleaved passenger strand. The lower panel is a digital over-exposure of the region of the gel corresponding to the 9-nt passenger-strand cleavage product. The sizes of passenger strand non-specific hydrolysis products are indicated for reference. These hydrolysis products were present before incubation in lysate in the total siRNA population, but not in the sample that had been purified by

native gel electrophoresis. The 9-nt passenger-strand cleavage product bears a 3´ hydroxyl group, whereas hydrolysis is expected to yield a mixture of 3´, 2´, and 2´,3´cyclic phosphate termini. Thus, the 9-nt cleavage product migrates between the 9- and 10-nt long hydrolysis products. (C) Passenger-strand 5´ cleavage product is disproportionately stable early in RISC assembly, consistent with its still being bound to the guide strand. Sod1 siRNA containing 5´-32P-labeled passenger was incubated in a standard in vitro RNAi reaction, in the absence or presence of a 2´-*O*-methyl oligonucleotide complementary to the passenger strand, and the fraction of siRNA passenger strand recovered as a 9-nt cleavage product was plotted versus time.

pathway: a double-stranded siRNA bearing a nick on the passenger strand. To determine if such a species exists, we assembled RISC in vitro using an siRNA containing a radiolabeled passenger strand. At each time point, a sample of the reaction was deproteinized at room temperature in the presence of a 20-fold excess of a 2´-*O*-methyl oligo nucleotide containing at its center the 21-nt sequence of the guide-strand RNA, designed to prevent reannealing of the original passenger strand with the guide strand. Next, the reaction was analyzed by native gel electrophoresis. Like the gel system used in Figure III-3E, this gel system could resolve the double-stranded siRNA from the two radiolabeled RNA heteroduplexes (the heteroduplex formed between the 2´-*O*-methyl oligonucleotide and the 9-nt passenger-strand cleavage product and the heteroduplex formed between the 2´-*O*-methyl oligonucleotide and the full-length passenger strand). During the first five minutes of the reaction, most of the labeled passenger strand migrated in the native gel as double-stranded siRNA, although some 9-nt passenger-strand cleavage fragment released from the siRNA duplex was also detected (Supplemental Figure III-2).

To examine whether some of the double-stranded RNA represented the nicked duplex predicted by the model, the double-stranded siRNA population was eluted from the native gel and analyzed by denaturing gel electrophoresis to resolve full-length passenger strand from the 9-nt, 5´ passenger-strand cleavage fragment (Figure III-4B). For comparison, the total reaction was analyzed in parallel. We found that at early times in RISC assembly, the double-stranded siRNA was a mixture of guide strand bound to full-length passenger strand (species *1* in Figure III-4A) and cleaved passenger strand

(species *2* in Figure III-4A). Moreover, a 2´-*O*-methyl oligonucleotide trap intended to capture and protect the 9-nt cleavage product increased only slightly the amount of 9-mer recovered at early times (≤ 5 min) in RISC assembly, but dramatically increased the recovery of this species later (≥ 10 min) (Figure III-4C and Supplemental Figure III-1B). These data suggest that early in RISC assembly, the 9-nt fragment of the passenger strand is protected from degradation even in the absence of the 2´-*O*-methyl oligonucleotide trap. The simplest explanation is that early in RISC assembly the 9-nt passenger-strand fragment remains bound to the guide strand, in the target RNA-binding cleft of Ago2, and is therefore naturally protected from nucleolytic destruction.

A Bypass Mechanism for siRNA Unwinding

Our data suggest that, under normal conditions, passenger-strand cleavage is the dominant mechanism for initiating siRNA strand dissociation during the production of Ago2-containing RISC. To assess more quantitatively the contribution of passenger strand cleavage to Ago2 RISC assembly, we sought an siRNA chemical modification that would inhibit passenger strand cleavage while leaving intact the essential structure of the siRNA. Phosphorothioate modification of the scissile phosphodiester bond inhibits Ago2-catalyzed cleavage of an mRNA target, and this inhibition is partially but not fully rescued by Mn^{2+} (Schwarz et al., 2004; Rivas et al., 2005). We surmised that a single phosphorothioate at the scissile phosphate of the passenger strand of an siRNA should inhibit passenger-strand cleavage (Figure III-5A). Phosphorothioate linkages are chiral, with sulfur substitution of each of the two non-bridging oxygens designated Rp or Sp.

Figure III-5

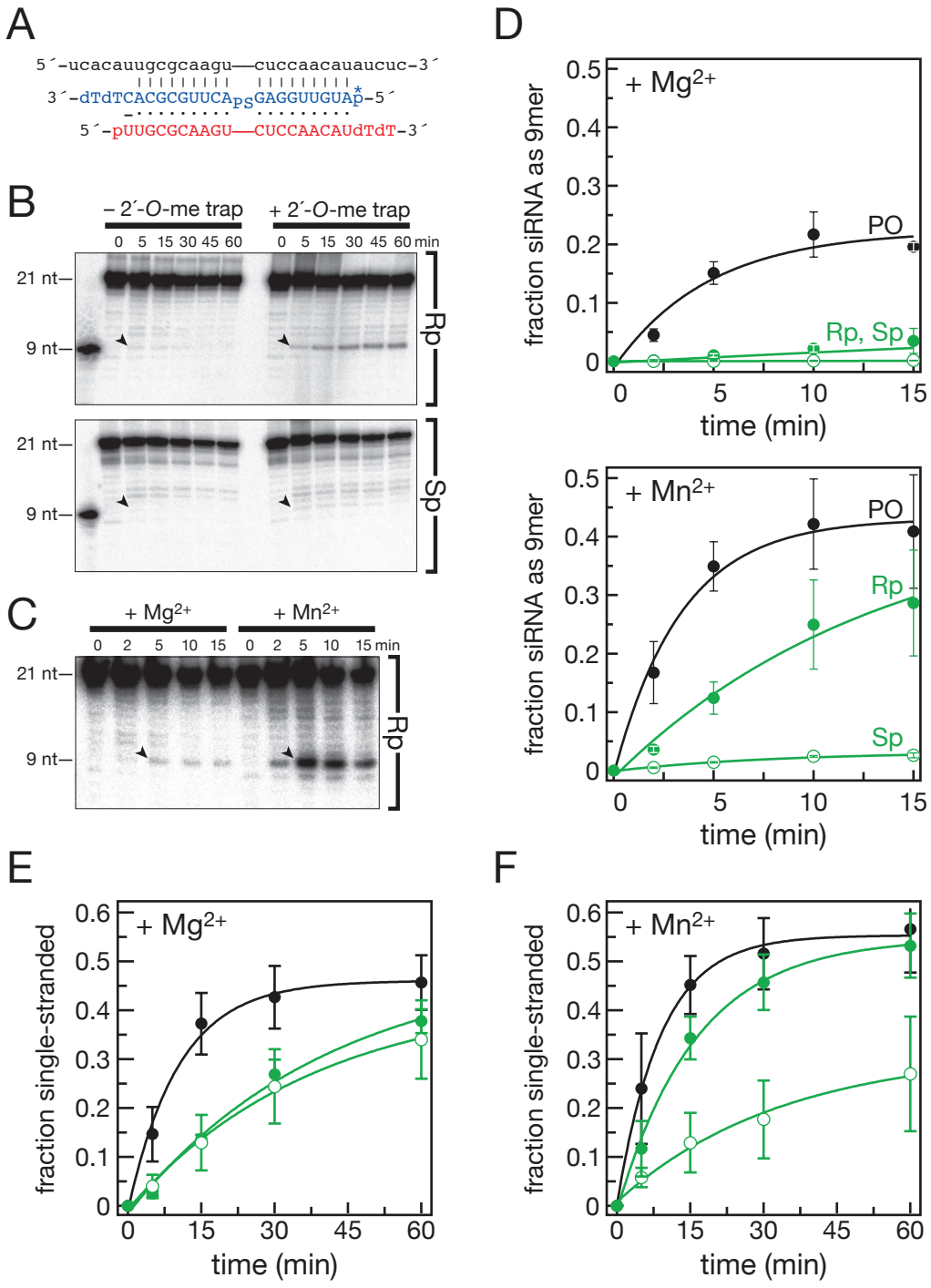

A
5´-ucacauugcgcaagu—cuccaacauaucuc-3´
3´-dTdTCACGCGUUCAPSGAGGUUGUAp*-5´
5´-pUUGCGCAAGU—CUCCAACAUdTdT-3´
B
– 2´-O-me trap
+ 2´-O-me trap
0 5 15 30 45 60 0 5 15 30 45 60 min
21 nt
9 nt
Rp
Sp
C
+ Mg2+
+ Mn2+
0 2 5 10 15 0 2 5 10 15 min
21 nt
9 nt
Rp
D
+ Mg2+
PO
Rp, Sp
fraction siRNA as 9mer
time (min)
+ Mn2+
PO
Rp
Sp
fraction siRNA as 9mer
time (min)
E
+ Mg2+
fraction single-stranded
time (min)
F
+ Mn2+
fraction single-stranded
time (min)

Figure Legend III-5. A phosphorothioate (PS) linkage at the siRNA scissile phosphate inhibits passenger cleavage and slows dissociation of the two siRNA strands. (A) siRNA were synthesized with a single PS modification at the passenger-strand scissile phosphate and the diastereomers resolved by reverse-phase HPLC (Supplemental Figure III-4). (B) Both Rp and Sp PS diastereomers inhibited passenger-strand cleavage. When a 2´-*O*-methyl oligonucleotide complementary to the passenger was included in the reaction, small amount of 9-nt passenger-strand cleavage product was detected for the Rp, but not the Sp, passenger strand. (C) The inhibitory effect of the Rp phosphorothioate linkage could be overcome when the thiophilic divalent cation Mn2+ was included in the reaction. A 2´-*O*-methyl oligonucleotide was not included in this experiment. (D) The rate of passenger-strand cleavage was measured in the presence of a 2´-*O*-methyl oligonucleotide trap for the all-phosphodiester (PO), Rp and Sp PS siRNAs in the presence of 2.5 mM supplemental Mg^{2+} or Mn^{2+}. (E) and (F) The rate of production of single-stranded guide siRNA was measured using a radiolabeled guide strand for siRNAs containing a phosphodiester, Rp or Sp PS-modified passenger strand in the presence of supplemental Mg^{2+} or Mn^{2+}. In (D), (E) and (F), each data point is the average of three trials ± standard deviation.

Consistent with the idea that both target-RNA and siRNA passenger-strand cleavage are catalyzed by the Mg^{2+}-dependent, endonuclease activity of Ago2, a racemic mixture of Rp and Sp phosphorothioate linkages between nucleosides 9 and 10 of the siRNA passenger strand decreased the rate of siRNA strand separation by ~3-fold, relative to the rate for the all-phosphodiester siRNA or to phosphorothioate substitution at the adjacent passenger-strand phosphodiester bonds (Supplemental Figure III-3).

We resolved the mixture of Rp and Sp phosphorothioate-substituted siRNA passenger strands by reverse-phase HPLC and prepared two diastereomerically pure siRNA duplexes, one with an Rp phosphorothioate and one with an Sp phosphorothioate at the passenger strand scissile phosphate (Supplemental Figure III-4). Each siRNA was examined for passenger-strand cleavage during incubation in a standard RISC assembly reaction, in the absence and the presence of a 2´-*O*-methyl oligonucleotide trap designed to protect the 9-nt passenger-strand cleavage fragment from degradation (Figure III-5B). Substitution of the scissile phosphate of the passenger strand of the siRNA duplex reduced the rate of 9-mer production ~10-fold with the Rp diastereomer and >30-fold with the Sp diastereomer (Figure III-5B and data not shown). Inhibition of endonucleolytic cleavage by a phosphorothioate linkage at the scissile phosphate can be partially rescued by supplementing the reaction with Mn^{2+}, a thiophilic cation (Verma and Eckstein, 1998; Schwarz et al., 2004). Similarly, supplementing the RISC assembly reaction with 2.5 mM Mn^{2+}, but not Mg^{2+}, rescued cleavage of the Rp phosphorothioate-containing passenger strand (Figure III-5C, D). Mn^{2+} did not detectably rescue passenger-strand cleavage for the Sp phosphorothioate-substituted siRNA (Figure III-5D).

Supplemental Figure III-5 shows for a second siRNA of unrelated sequence that both Rp and Sp phosphorothioates at the scissile phosphate of the passenger strand inhibited passenger strand cleavage, but that only the Rp diastereomer was rescued by supplemental Mn^{2+}. Together, these data are consistent with the partial rescue by Mn^{2+} of Ago2-directed cleavage of a target RNA substituted with a racemic mixture of phosphorothioate diastereomers at the scissile phosphate (Schwarz et al., 2004; Rivas et al., 2005).

The production of single-stranded guide strand from the siRNA duplex, a measure of mature RISC production, was slower for an siRNA containing an Rp or Sp phosphorothioate at the scissile phosphate of the passenger strand relative to an all phosphodiester passenger strand (Figure III-5E). When supplemental Mn^{2+} was provided, the production of single-stranded guide strand was enhanced for the Rp phosphorothioate-substituted siRNA, relative to the unmodified siRNA, but not for the Sp (Figure III-5F). In Figure III-5E, supplemental Mg^{2+} was included so that the divalent cation concentration was equivalent to the total divalent cation concentration when supplemental Mn^{2+} was added. The higher-than-standard Mg^{2+}, which was likely much greater than the intracellular free Mg^{2+} concentration, enhanced RISC assembly, as monitored by the production of single-stranded guide (Figure III-5E) and RISC activity (data not shown), relative to that observed for the phosphodiester and phosphorothioate substituted siRNAs under standard conditions.

Together, the results in Figure III-5 provide additional evidence for the model of cleavage-assisted RISC assembly (Figure III-4A). Substitutions within the guide-strand

backbone known to inhibit Ago2-catalyzed RNA cleavage inhibit both passenger-strand cleavage and maturation of the RISC. Divalent metal ions that partially rescue cleavage partially rescue RISC maturation. Nonetheless, when viewed quantitatively, the results of these experiments, which were performed at a non-standard, high divalent cation concentration, suggest that inhibition of passenger-strand cleavage reduced RISC assembly but did not prevent it. That is, using an siRNA duplex for which very little cleavage product could be detected (Figure III-5D) resulted in a reduced but readily detectable amount of mature RISC, as measured by single-stranded guide formation (Figure III-5E). Part of this difference can be explained by the finite lifetime of the 9-mer cleavage product, even in the presence of the 2´-*O*-methyl oligonucleotide trap. However, we suspect that much of this difference is due to a bypass mechanism for RISC maturation that is more efficient at elevated divalent cation concentrations. The existence of such a bypass mechanism implies that passenger-strand cleavage is the normal mechanism by which siRNA strand separation is initiated, but it is not obligatory.

How big a role does passenger-strand cleavage play in the production of active, Ago2-containing RISC for siRNAs? To assess the effect of blocking passenger-strand cleavage during RISC assembly, we compared the amount of RISC activity produced for the all-phosphodiester siRNA and the two diastereomerically-pure phosphorothioate siRNAs after 5 and after 10 min of RISC assembly (Figure III-6). RISC was assembled in a standard reaction for 5 or 10 min, quenched by treatment with *N*-ethylmaleimide, and then a radiolabeled target RNA was added and target cleavage monitored. When RISC was assembled for just 5 min, considerable RISC was produced from the all

Figure III-6

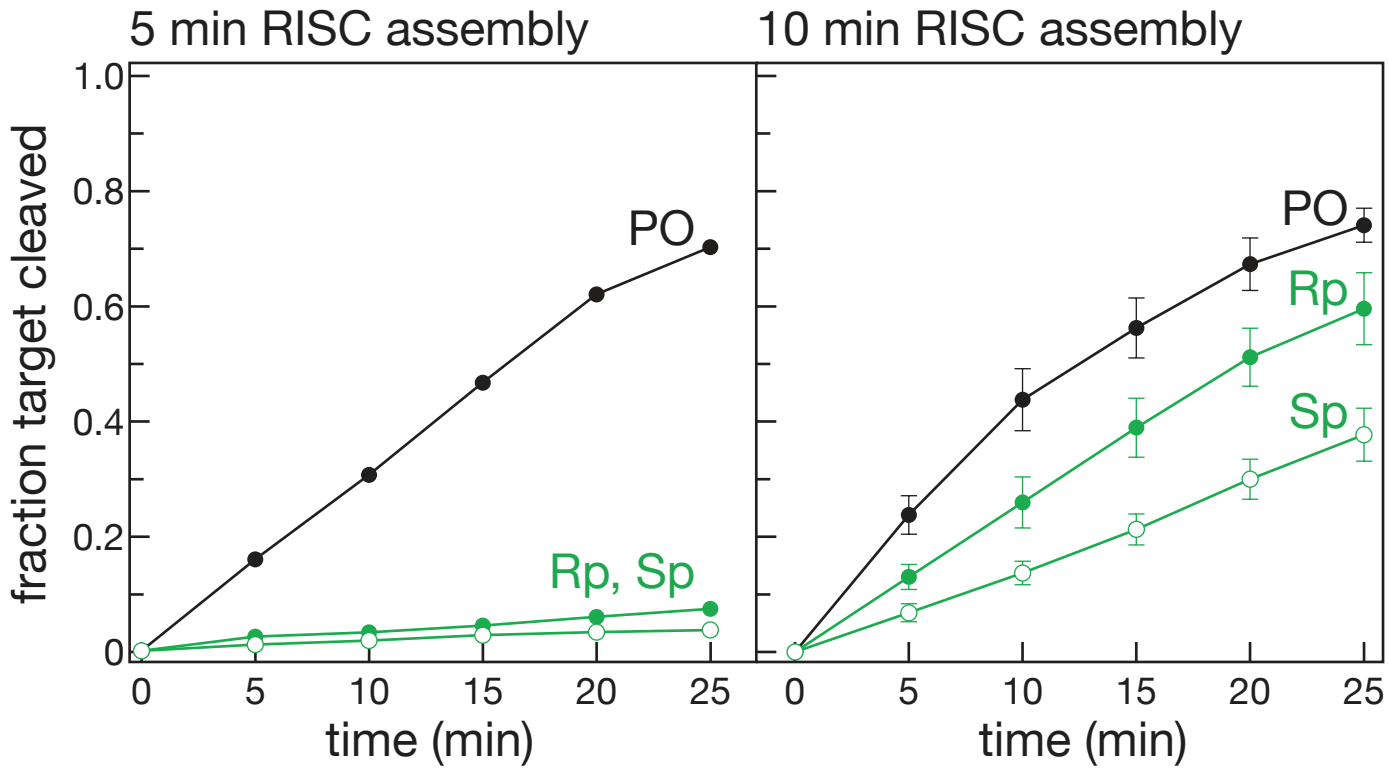


Figure Legend III-6. Passenger-strand cleavage is required for rapid RISC assembly, but a slower, bypass mechanism dissociates the two siRNA strands when passenger-strand cleavage is inhibited. The assembly of active RISC was assayed by measuring the production of target-cleaving activity. RISC was assembled for 5 or 10 min, quenched, then a cap-radiolabeled target RNA was added to the reaction and the rate of target cleavage monitored.

phosphodiester siRNA, but little or no RISC activity was detected for the two siRNAs bearing an Rp or an Sp phosphorothioate at the passenger strand scissile phosphate. In contrast, when RISC was assembled for 10 min, RISC activity was detected for all three siRNAs. The all-phosphodiester siRNA was most active; the siRNA bearing an Rp phosphorothioate at the passenger-strand scissile phosphate produced an intermediate amount of RISC activity; the Sp siRNA showed the least activity. These results show that RISC activity is rapidly assembled when the passenger strand can be cleaved. When it cannot be cleaved, RISC is slower to form, because it must rely on a bypass mechanism in which the passenger strand is dissociated intact from the guide. Because the bypass mechanism is so much slower than the cleavage-assisted mechanism, we conclude that nearly all of the Ago2 RISC generated with a standard siRNAs duplex is formed through the cleavage-assisted mechanism.

Passenger-strand cleavage Facilitates Assembly of Human Ago2 RISC

The RISC assembly pathway has been studied largely in extracts from *Drosophila* but is presumed to be similar in human cells, in which an RLC-like complex has recently been proposed to load RISC containing Ago2 (Chendrimada et al., 2005). Because Ago2 is the only human Argonaute protein with known cleavage activity, this silencing complex is thought to be the one that mediates target-strand cleavage in humans (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). Does passenger-strand cleavage play a role in human Ago2 RISC assembly? Supporting the view that it does, the passenger strand, but not the guide, of an siRNA duplex in which the guide strand corresponds to miR-1 was

Figure III-7

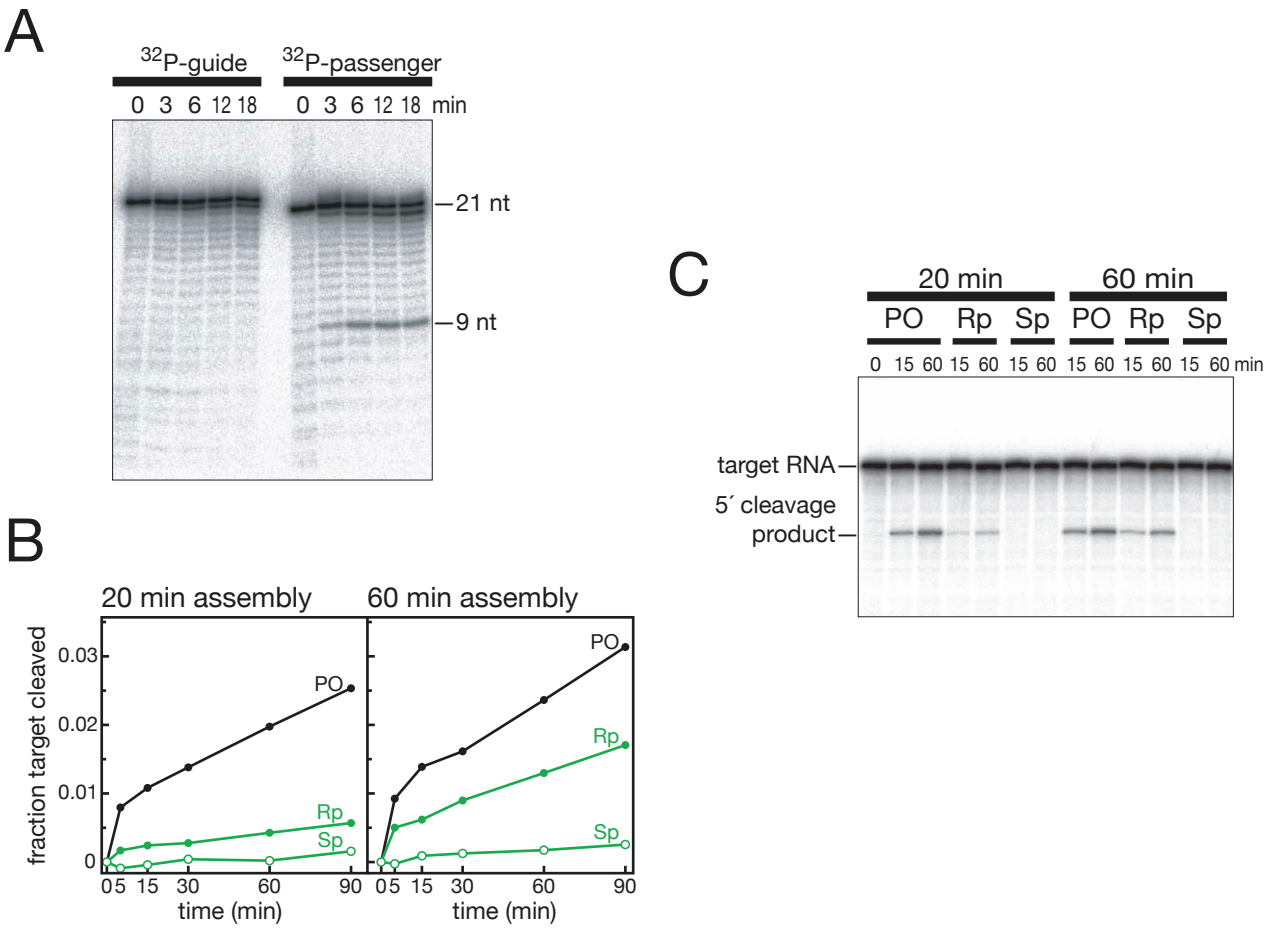


Figure Legend III-7. Passenger-strand cleavage is required for rapid assembly of the Ago2- containing human RISC. (A) The passenger strand, but not the guide, is cleaved when human RISC is assembled. An siRNA in which the guide strand corresponded to miR-1 was used. A 2′-*O*-methyl oligonucleotide complementary to the 5′-^{32}P-radiolabeled strand was included in each reaction. (B) and (C) All-phosphodiester (PO), Rp, or Sp phosphorothioate (PS)-containing sod1 siRNA (diagrammed in Figure 5A) were used to program RISC assembly in human HeLa cell S100 extract for 20 or 60 min, then RISC assembly was quenched, a target RNA added, and the production of Ago2-containing RISC activity measured by monitoring target cleavage.

cleaved when incubated in human HeLa cell S100 extract under conditions that support RNAi in vitro (Figure III-7A). As observed for RISC assembly in *Drosophila* embryo lysate, a racemic mixture of Rp and Sp phosphorothioate linkages between nucleosides 9 and 10 of the passenger strand inhibited RISC assembly in human cell extracts, and this inhibition was substantially greater with phosphorothioate substitution at this position compared to substitution of the adjacent phosphodiester bonds (Supplemental Figure III-6). Furthermore, assembly was inhibited by both the Sp and Rp diastereomers but far more by the Sp than by the Rp, as was characteristic of the *Drosophila* RISC (Figure III-7B and C). In this experiment, RISC was assembled for 20 or 60 min with the SOD1 siRNAs used in Figure III-5. Then assembly was quenched with *N*-ethylmaleimide, and a radiolabeled SOD1 RNA was added to the reaction in order to monitor target cleavage as a measure of Ago2 RISC activity. Thus, multiple lines of evidence support the conclusion that passenger-strand cleavage facilitates assembly of the human Ago2 RISC, just as it does for the *Drosophila* Ago2 RISC. Indeed, the virtually undetectable assembly with the Sp substitution, even after a 60-minute assembly incubation (Figure III-7B and C), implicates the cleavage-assisted mechanism for nearly all of the Ago2 RISC assembly occurring with a standard siRNA duplex.

The Bypass Mechanism Appears Sufficient for Assembling MicroRNAs

If siRNAs are assembled into human Ago2 RISC by a passenger-strand cleavage assisted mechanism, how are miRNAs assembled? To begin to address this question we examined assembly of the muscle-specific miRNA, miR-1, which is not present in HeLa

cells. We prepared three small RNA duplexes (Figure III-8A) predicted to load miR-1 into RISC: (1) a miR-1/miR-1* duplex, like that predicted to result from Dicer processing of pre-miR-1 from the human mir-1-1 gene; (2) a functionally asymmetric siRNA in which miR-1 was paired to antisense miR-1 with a 'frayed' miR-1 5´ end (miR-1 siRNA); and (3) an siRNA in which miR-1 was paired to antisense miR-1, but for a G:U wobble at position 2 of miR-1 (miR-1 'GU' siRNA). All three duplexes mediated efficient Ago2 RISC loading, as measured functionally by target cleavage (Figure III-8B, PO time courses). For each duplex, we synthesized a passenger strand with a racemic phosphorothioate at the scissile phosphate, i.e. at the linkage spanning the nucleotides that pair to nucleotides 10 and 11 of miR-1 (Figure III-8A). Substitution of the passenger-strand scissile phosphate with the phosphorothioate strongly inhibited the assembly of active RISC for the miR-1 siRNA, had a more modest effect for the miR-1 'GU' siRNA, and had no effect for the miR-1/miR-1* duplex. These data suggest that passenger-strand cleavage facilitates RISC assembly for the standard miR-1 siRNA, plays a supportive role for the miR-1 'GU' siRNA, but plays no role for the miR-1/miR-1* duplex. Analogous results were obtained when miR-1 was presented as the authentic pre-miRNA hairpin or in the context of a perfectly paired short-hairpin RNA (shRNA); again, the phosphorothioate inhibited assembly when the passenger strand was fully paired to miR-1 but not when it was within the miR-1/miR-1* duplex (Figure III-8A and C). These results support the idea that miRNAs, when paired to their natural passenger strands, the miRNA* strands, are loaded into the Ago2 RISC without cleavage of the miRNA*. This preference for the bypass mechanism is because of the multiple mismatches typical of

Figure III-8

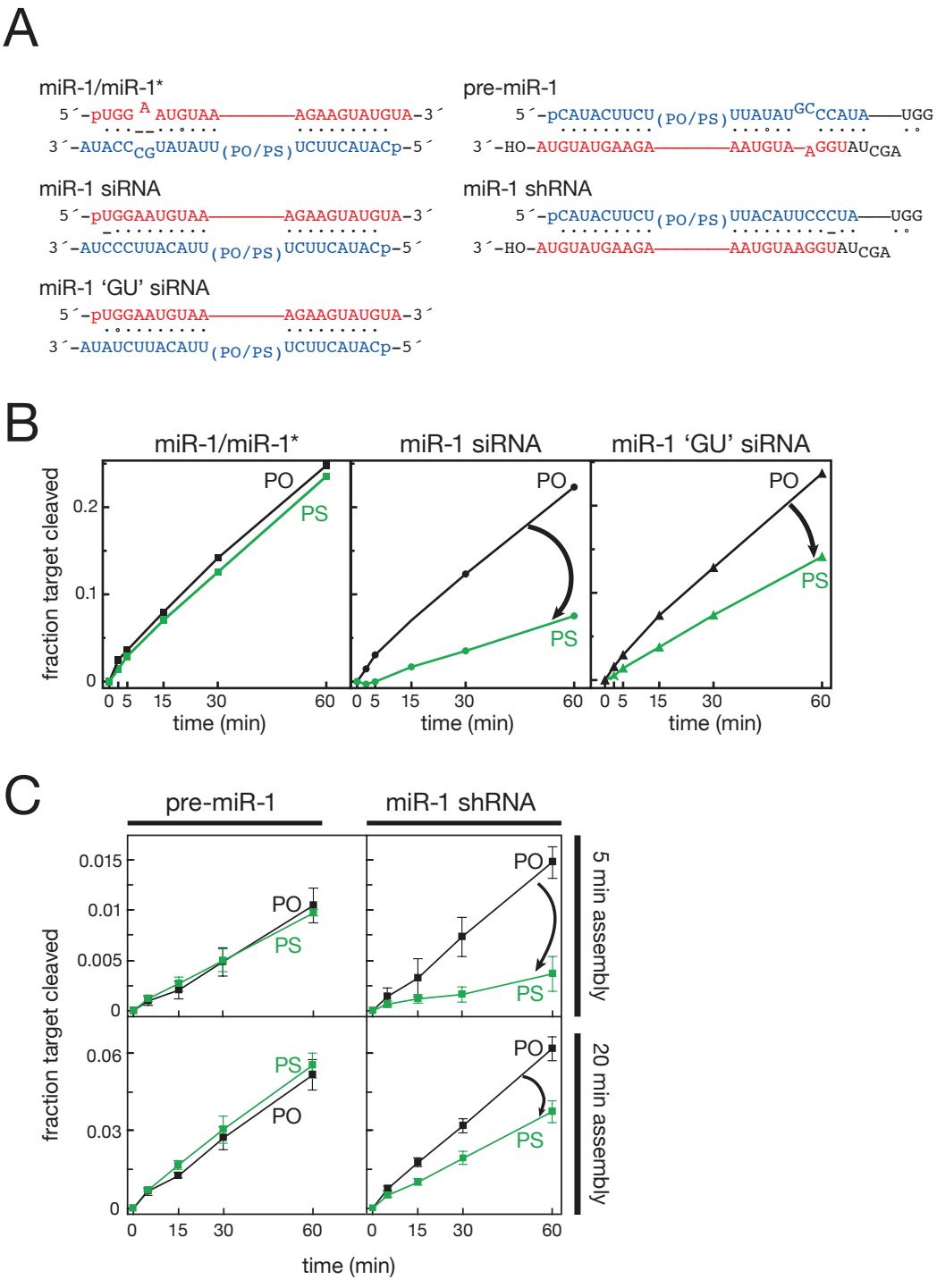

A
miR-1/miR-1*
5´-pUGG A AUGUAA——————AGAAGUAUGUA-3´
3´-AUACC CG UAUAUU (PO/PS) UCUUCAUACp-5´
miR-1 siRNA
5´-pUGGAAUGUAA——————AGAAGUAUGUA-3´
3´-AUCCCUUACAUU (PO/PS) UCUUCAUACp-5´
miR-1 'GU' siRNA
5´-pUGGAAUGUAA——————AGAAGUAUGUA-3´
3´-AUAUCUUACAUU (PO/PS) UCUUCAUACp-5´
pre-miR-1
5´-pCAUACUUCU (PO/PS) UUAUAU GC CCAUA——UGG
3´-HO-AUGUAUGAAGA——————AAUGUA—A GGU AU CGA
miR-1 shRNA
5´-pCAUACUUCU (PO/PS) UUACAUUCCCUA——UGG
3´-HO-AUGUAUGAAGA——————AAUGUAAGGU AU CGA
B
miR-1/miR-1*
miR-1 siRNA
miR-1 'GU' siRNA
fraction target cleaved
0
0.1
0.2
PO
PS
0 5 15 30 60
time (min)
C
pre-miR-1
miR-1 shRNA
fraction target cleaved
0
0.005
0.01
0.015
0
0.03
0.06
PO
PS
0 15 30 60
time (min)
5 min assembly
20 min assembly

Figure Legend III-8. Phosphorothioate modification of the scissile phosphate of a miR-1 siRNA passenger strand and of the *pro*-passenger strand of a miR-1 shRNA, but not of the miR-1* strand of a miR-1/miR-1* duplex nor of *pro*-miR-1* of authentic pre-miR-1-1, inhibited the production of active miR-1-programmed RISC. (A) siRNAs and hairpin RNAs used in this study. 'PO/PS' indicates linkages at which the effect of a racemic phosphorothioate modification was compared to a phosphodiester bond. (B) The three siRNAs in (A) were prepared either with a phosphodiester linkage (PO) or a phosphorothioate (PS) at the passenger-strand scissile phosphate. RISC was assembled for 5 min, quenched, then a cap-radiolabeled RNA added and the production of Ago2-containing RISC measured by monitoring target cleavage. The phosphorothioate inhibits assembly (arrows) of active Ago2-containing RISC most strongly for the miR-1 siRNA, less so for the miR-1 'GU' siRNA. No phosphorothioate inhibition was detected for the miR- 1/miR-1* duplex, suggesting that passenger-strand cleavage is important for the rapid assembly of Ago2 RISC for siRNAs, but not for at least some miRNAs. (C) Target cleavage activity was assayed using the hairpin precursors diagrammed in (A). RISC was programmed using a either pre-miR-1 RNA containing all phosphodiester (PO) linkages or a single, racemic phosphorothioate (PS) linkage at the predicted scissile phosphate of *pro*-miR-1* or was programmed using a miR-1 shRNA with all PO linkages or a PS linkage at the predicted scissile phosphate for the *pro*-passenger strand. HeLa S100 extract was programmed for the indicated times, quenched with *N*-ethylmaleimide, and then a 32P-radiolabeled target RNA containing a 21 nt sequence complementary to miR-1 was added.

miRNA/miRNA* duplexes, which presumably inhibit the cleavage step of the cleavage-assisted mechanism while accelerating the bypass mechanism, obviating the need for guide-strand cleavage to enhance strand separation.

Discussion

Our data suggest a new model for Ago2 RISC assembly in which siRNAs are initially loaded into Ago2 as duplexes, and then Ago2 cleaves the passenger strand of the siRNA, facilitating its displacement and leaving the siRNA guide strand bound stably to Ago2 (Figure III-4A). In support of this cleavage-assisted assembly model, we find that the passenger strand is cleaved during RISC assembly. The position of the cleavage site on the passenger strand and the stereospecificity of inhibition by phosphorothioates are both diagnostic of siRNA-directed, Ago2-catalyzed cleavage (Figures III-1, 5 and 7). Both Ago2 and the RLC are required for this cleavage (Figure III-2), and as anticipated by the model, cleavage occurs before the passenger strand has dissociated from the guide strand, that is, before the formation of active RISC (Figure III-3).

A cleavage-assisted assembly mechanism is also consistent with previous data. For example, it is consistent with our earlier finding that early (5 min) in assembly, RISC contains considerable amounts of double-stranded siRNA (Tomari et al., 2004b). When the asymmetry rules for siRNA loading were first uncovered, they evoked the idea of a non-processive helicase that separated the two strands of the duplex, starting from the end that was less-stably paired (Khvorova et al., 2003; Schwarz et al., 2003). However, subsequent studies showed that the R2D2/Dicer heterodimer, the core of the RLC, binds

asymmetrically to double-stranded siRNAs (Tomari et al., 2004b), suggesting that a nonprocessive helicase does not sense siRNA thermodynamic asymmetry during RISC loading. Unlike Ago2 loaded via the RISC assembly pathway, affinity-purified (Liu et al., 2004) or recombinant Ago2 (Rivas et al., 2005) can only be programmed with single-, not double-stranded, siRNA, suggesting that the essential function of the RLC is to facilitate the directional loading of a double-stranded siRNA into Ago2. Such directional loading would place the 5´ end of the siRNA guide strand, in the context of a double stranded siRNA, into the phosphate-binding pocket of Ago2 (Ma et al., 2005; Parker et al., 2005). The idea that Ago2-mediated passenger-strand cleavage triggers siRNA strand dissociation also accounts for our previous observation that one passenger strand appears to be destroyed for every cycle of assembly of a guide strand into target-cleaving RISC (Schwarz et al., 2003). Passenger-strand cleavage would strongly reinforce siRNA functional asymmetry by coupling passenger-strand destruction to RISC assembly.

Blocking Ago2-mediated passenger-strand cleavage by substituting a phosphorothioate for the scissile phosphate revealed a slower, bypass mechanism that dissociates the two siRNA strands. Of course, phosphorothioate-substituted siRNAs do not occur in nature, but miRNA/miRNA* duplexes do often contain central mismatches predicted to block cleavage of the miRNA* strand, which is the analog of the siRNA passenger strand (Bartel, 2004). Most, if not all, miRNAs are efficiently loaded into Ago2 in cultured human cells (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005), and miR-127, miR-136, miR-196, miR-431, miR-433, and miR-434 are known to cleave their targets in vivo (Yekta et al., 2004; Davis et al., 2005), and are therefore presumed to

function in an Ago2-containing RISC. Likewise, at least some miRNAs are loaded into Ago2 in cultured *Drosophila* cells (Förstemann et al. 2007).

We envision that in the bypass pathway—as in the cleavage-assisted pathway—Argonaute proteins are loaded with double-, rather than single-stranded siRNAs. Dissociation of the full-length passenger strand would then require breaking is interaction with the "seed" region of the guide strand (nucleotides 2-7), a region proposed to mediate miRNA target pairing (Lewis et al., 2003; Bartel, 2004). For metazoan miRNAs, conserved Watson-Crick pairing to the seed is necessary and sufficient for accurate target prediction (Lewis et al., 2003; Lewis et al., 2005; Brennecke et al., 2005; Krek et al., 2005). Similarly, Watson-Crick pairing to the seed can be sufficient for miR-19 mediated repression (Doench and Sharp, 2004; Lim et al., 2005; Brennecke et al., 2005), and pairing to this 5´ region of the guide strand makes a far greater contribution to target binding affinity than does pairing to the 3´ end (Haley and Zamore, 2004). The tight binding to the 5´ portion of the guide strand is what presumably prevents an appreciable amount of a standard siRNA duplex from being assembled into Ago2 through the bypass mechanism. In contrast, when presented to Ago2 as a miR-1/miR-1* duplex, miR-1 loads efficiently without need for passenger-strand cleavage, because a Watson-Crick pairing to the miRNA seed is disrupted (Figure III-8). Indeed, far less disruption of seed pairing might be sufficient to enable the bypass mechanism to begin to play a substantial role, as hinted by our results using the miR-1 'GU' siRNA, which has a single G:U wobble disrupting perfect Watson-Crick pairing to the seed (Figure III-8B). Passenger strand cleavage appeared less important for this duplex than for the standard siRNA duplex

(Figure III-8B), a result consistent with computational and experimental studies of miRNA target specificity imply that single G:U wobble pairs in the seed disproportionately perturb small RNA binding (Lewis et al., 2003; Doench and Sharp, 2004; Lewis et al., 2005; Brennecke et al., 2005).

In addition to loading miRNAs, the bypass mechanism might be used to load siRNAs into Argonaute proteins that have lost their catalytic amino acids, such as human Ago1, Ago3, and Ago4 (Meister et al., 2004b; Liu et al., 2004; Rivas et al., 2005). Conversely, *Arabidopsis thaliana* Argonaute1, a miRNA-guided plant Argonaute protein with a functional endonuclease domain (Baumberger and Baulcombe, 2005; Qi et al., 2005), might be loaded by a passenger-strand cleavage-assisted pathway. Although human Ago1 and Ago3 can bind standard siRNAs, endogenous Ago1 and Ago3 cannot support siRNA-directed RNAi in Ago2-knockout mouse embryonic fibroblasts (Liu et al., 2004). The function of Ago1, Ago3, and Ago4-containing RISC is not yet known. Perhaps the capacity for loading small RNAs via the cleavage-assisted pathway confers a degree of specificity to the function of different silencing complexes, with Ago2 able to use a broader range of small RNAs than those Argonaute proteins incapable of RNA cleavage.

Experimental Procedures

General Methods

Drosophila Schneider 2 (S2) cell, embryo, and ovary lysates and in vitro RNAi assays were as described (Haley et al., 2003; Tomari et al., 2004a; Forstemann et al.,

2005). siRNAs were deprotected according to manufacturer's protocol (Dharmacon). RNAi reactions with HeLa S100 extract were incubated at 30°C (Schwarz et al., 2002) using 20 nM siRNA, 1 mM ATP, 0.2 mM GTP, 5 mM $MgCl_2$, 25 mM creatine phosphate, and 20 U/ml RNasin (Promega). RISC assembly was quenched with 10 mM (f.c.) *N*-ethylmaleimide (Pierce) for 10 min at 4°C, followed by 11 mM DTT for 10 min at 4°C before the addition of radiolabeled target RNA.

Passenger-Strand Cleavage

siRNA passenger strands were 5´-^{32}P-radiolabeled with T4 polynucleotide kinase, isolated from a denaturing gel, annealed to a slight excess of unlabeled, phosphorylated guide strand, then the duplex was isolated from a 15% native polyacrylamide gel. 10 nM radiolabeled siRNA was incubated in a standard in vitro RNAi reaction; the reactions were quenched by adding 2X Proteinase K buffer (200 mM Tris-Cl [pH 7.5], 25 mM EDTA, 300 mM NaCl, 2% [w/v] SDS), 2 mg/ml Proteinase K, and 1 μg glycogen, incubated for 30 min at 65°C, extracted with an equal volume of phenol/chloroform (1:1), and the RNA precipitated with 3 volumes absolute ethanol. RNAs were resolved by electrophoresis through a 20% denaturing (19:1) polyacrylamide gel, the gel dried under vacuum, and detected with a FLA 5000 phosphorimager (Fuji). To quantify the rate of passenger-strand cleavage, a 2´-*O*-methyl oligonucleotide (200 nM or 1 μM) complementary to the passenger strand was added to the reaction to stabilize the cleavage product.

Native and Two-dimensional RNA analysis

Production of single-stranded guide siRNA strand (Figures III-3, 5, and Supplemental Figure III-3) was measured as previously described (Nykänen et al., 2001; Okamura et al., 2004; Tomari et al., 2004b). For all native gel analyses, reactions were stopped with Proteinase K buffer (200 mM Tris-Cl, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% [w/v] sodium dodecylsulfate) containing 1.5 mM $Mg(OAc)_2$, 2 mg/ml Proteinase K, and 1 μg glycogen. After incubation for 30 min at 25°C, 3 volumes absolute ethanol were added, and the RNA allowed to precipitate for an additional 30 min at 25°C. The precipitates were collected by centrifugation, washed with 80% (v/v) ethanol, then dissolved in 2 mM Tris-Cl (pH 7.5), 3% (w/v) Ficoll-400, 0.04% (w/v) bromophenol blue, 100 mM KOAc, 30 mM HEPES, 2 mM $Mg(OAc)_2$, and resolved by electrophoresis through a 15% native polyacrylamide gel (19:1 acrylamide:bis; 89 mM Tris-Borate pH 8.3, 2 mM EDTA, 2.5 mM $Mg(OAc)_2$). In Supplemental Figure III-2 Mg^{2+} was not included in gel or buffer. For the two-dimensional analysis in Figure III-4B, the reactions were first resolved by native electrophoresis, the region of the gel corresponding to double-stranded siRNA was excised and the RNA eluted overnight in 2X Proteinase K buffer. The samples were then extracted with phenol:chloroform (1:1), precipitated with 3 volumes of ethanol, the precipitate collected by centrifugation, washed with 80% (v/v) ethanol, the pellets dissolved in 98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, and then resolved on a 20% denaturing urea-polyacrylamide sequencing gel.

Reverse-phase HPLC purification of phosphorothioate diastereomers

Twenty-one nt siRNA passenger strand containing a single phosphorothioate (PS) modification between nucleotides 9 and 10 was ethanol precipitated, resuspended in 0.1 M NH_4OAc (Buffer A), and 50 μg of RNA injected onto a Targa C18 HPLC column (Nest Group). Separation of the PS diastereomers was as described (*4, 5*) with slight modifications. The column was eluted with a 0-15% gradient of Buffer B (0.1 M NH_4OAc, 50% [v/v] acetonitrile) over 15 min followed by a 15-23% of Buffer B gradient over 45 min. Rp and Sp 21-mer retention times were approximately 24 min and 26 min, respectively. Purified diastereomer peak fractions were pooled, lyophilized, then redissolved in water and lyophilized again, for three cycles of lyophilization, flash-freezing in liquid nitrogen after each time dissolution in water. Sample recovery was about 50%. Diastereomer identity was established by nuclease P1 treatment. Samples were treated with 2 units nuclease P1 (US Biological) for 60 min at 37°C, dephosphorylated with calf intestinal phosphatase (New England Biolabs), and then the resulting nucleosides were separated on a Targa C18 HPLC column (Nest Group), run first in 0-8% Buffer B over 25 min, followed by elution with a 8-30% Buffer B over 35 min. The characteristic nuclease P1-resistant Rp CU dinucleotide eluted from the column at 33 min. To confirm the identity of the dinucleotide, chiral dinucleotide standards were prepared by separating a racemic mixture of a PS-modified CU dinucleotide (Dharmacon) by reverse phase-HPLC. The identities of the two peaks were confirmed by their resistance (Rp) or sensitivity (Sp) to digestion with nuclease P1. Integrity of the diastereomerically pure RNA was confirmed by electrospray mass spectrometry (Keck

Facility, Yale University).

Acknowledgements

We thank Dianne Schwarz and Ben Haley for help with target cleavage assays, Tingting Du and Alicia Boucher for assistance with fly husbandry, members of the Zamore lab for advice, suggestions, and critical comments on the text, and Bill Kobertz and Amos Heckendorf (Nest Group) for help with HPLC. PDZ is a W.M. Keck Foundation Young Scholar in Medical Research. This work was supported in part by grants from the National Institutes of Health to PDZ (GM62862 and GM65236) and DPB (GM67031), a post-doctoral fellowship from the Human Frontier Science Program to YT, and a post-doctoral fellowship from the Damon Runyon Cancer Research Foundation to CS.

Figure III-S1.

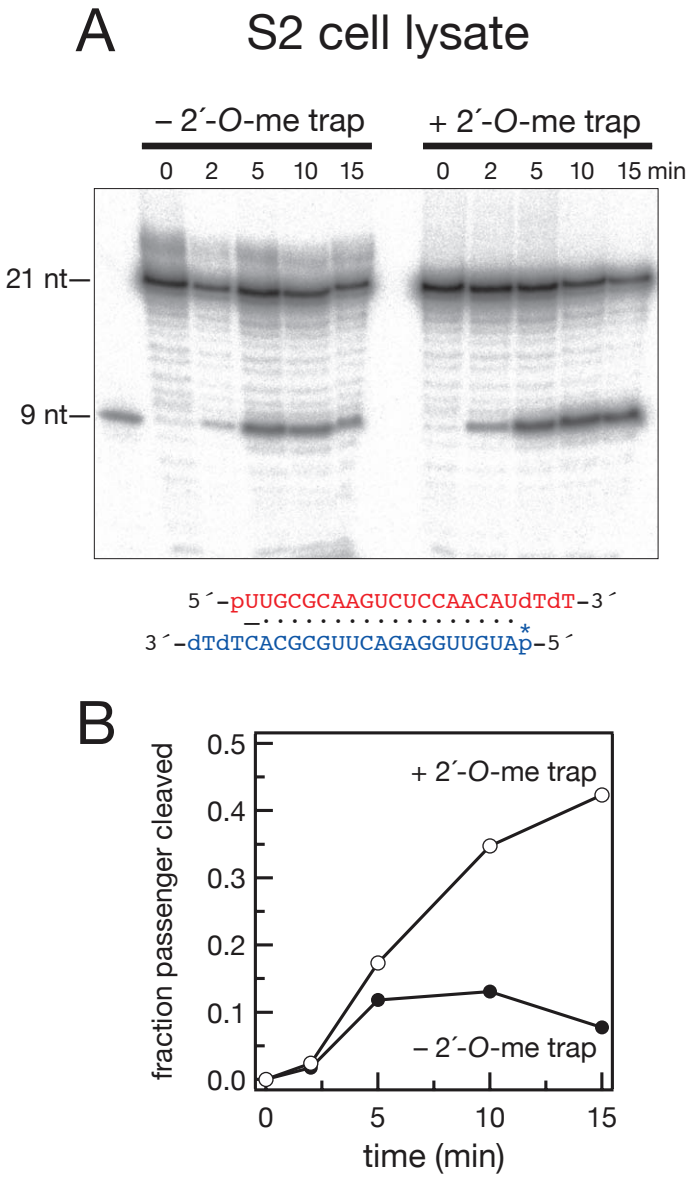


Figure Legend III-S1. Passenger-strand cleavage in Schneider 2 (S2) cell lysate. (A) The sod1 siRNA (Figure 1A), 5′-^{32}P-radiolabeled on the passenger strand, was incubated in an in vitro RNAi reaction prepared with *Drosophila* S2 cell lysate, in the absence or presenceof a 2′-*O*-methyl oligonucleotide complementary to the passenger strand. (B) Quantification of the data in (A).

Figure III-S2

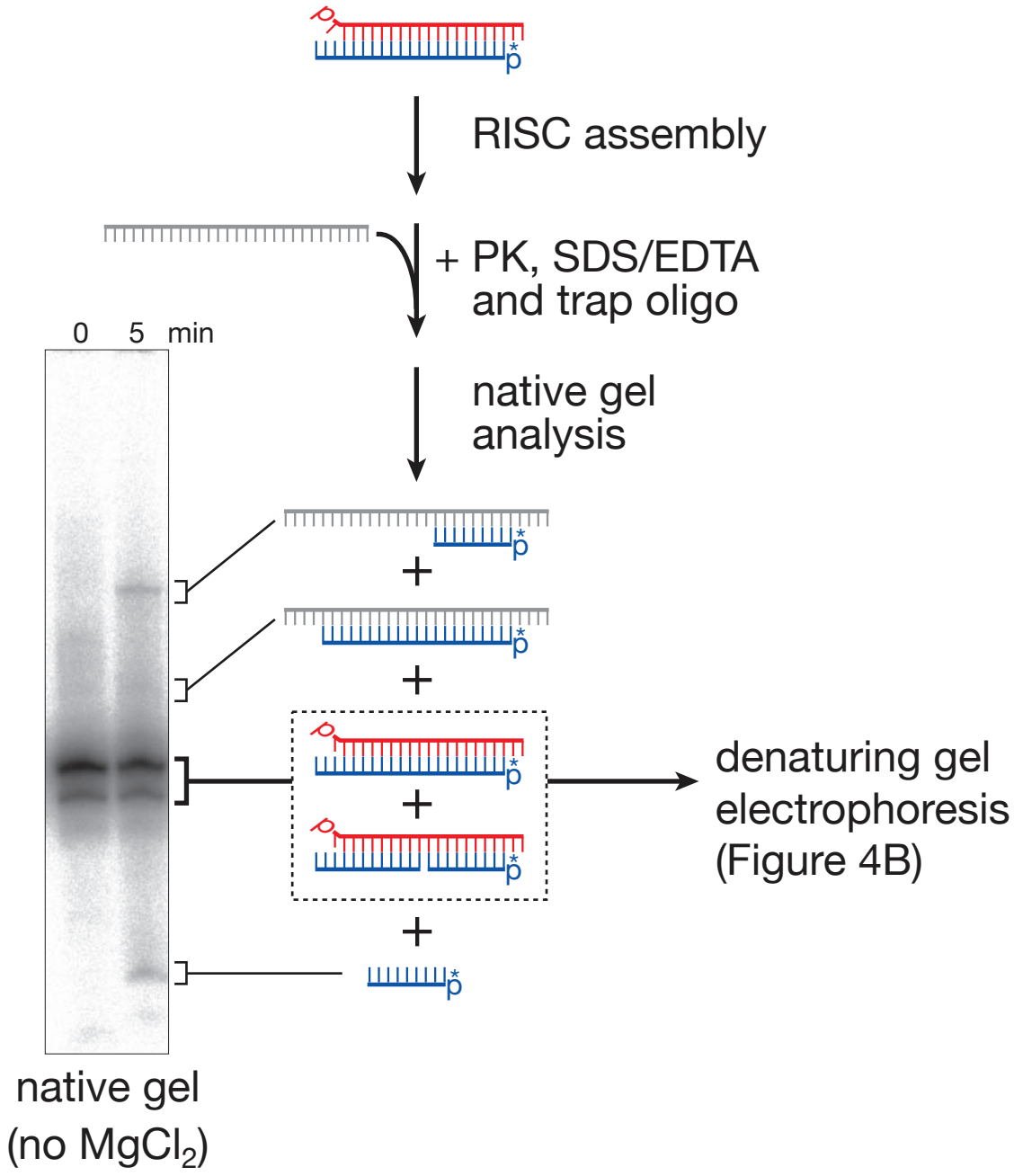


Figure Legend III-S2. Two dimensional analysis of double-stranded siRNA species. The sod1 siRNA, bearing a 5´-^{32}P-radiolabeled passenger strand, was incubated in a RISC assembly reaction with a 2´-*O*-methyl oligonucleotide complementary to the passenger strand. The identity of the bands was assigned by their comigration with synthetic RNA heteroduplexes as diagrammed. The species corresponding to double-stranded siRNA were excised from the gel and analyzed by denaturing polyacrylamide-urea gel electrophoresis. The denaturing gel analysis is shown in Figure 4B. Unlike that in Figure 3E, the native gel in this experiment did not contain Mg2+. Consequently, the heteroduplex formed between the 2´-*O*-methyl oligonucleotide and the 9-nt passenger strand cleavage product partially dissociates during electrophoresis, producing some free 9-nt, passenger-strand cleavage fragment.

Figure III-S3

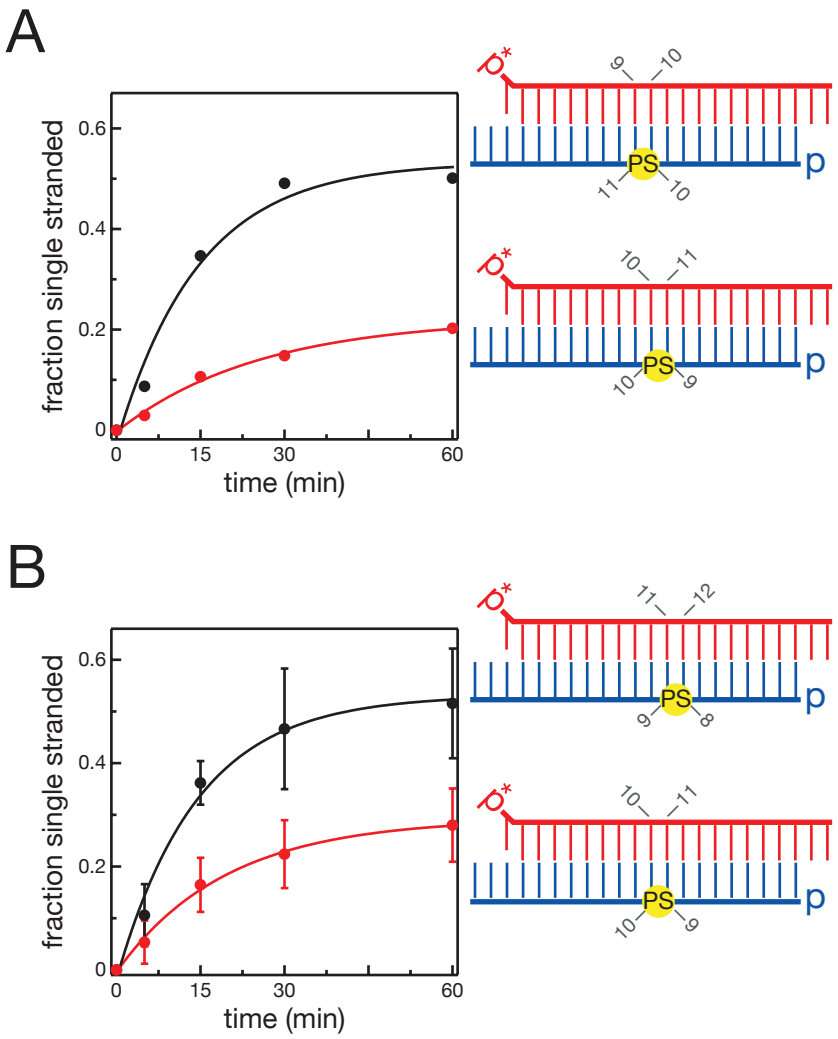
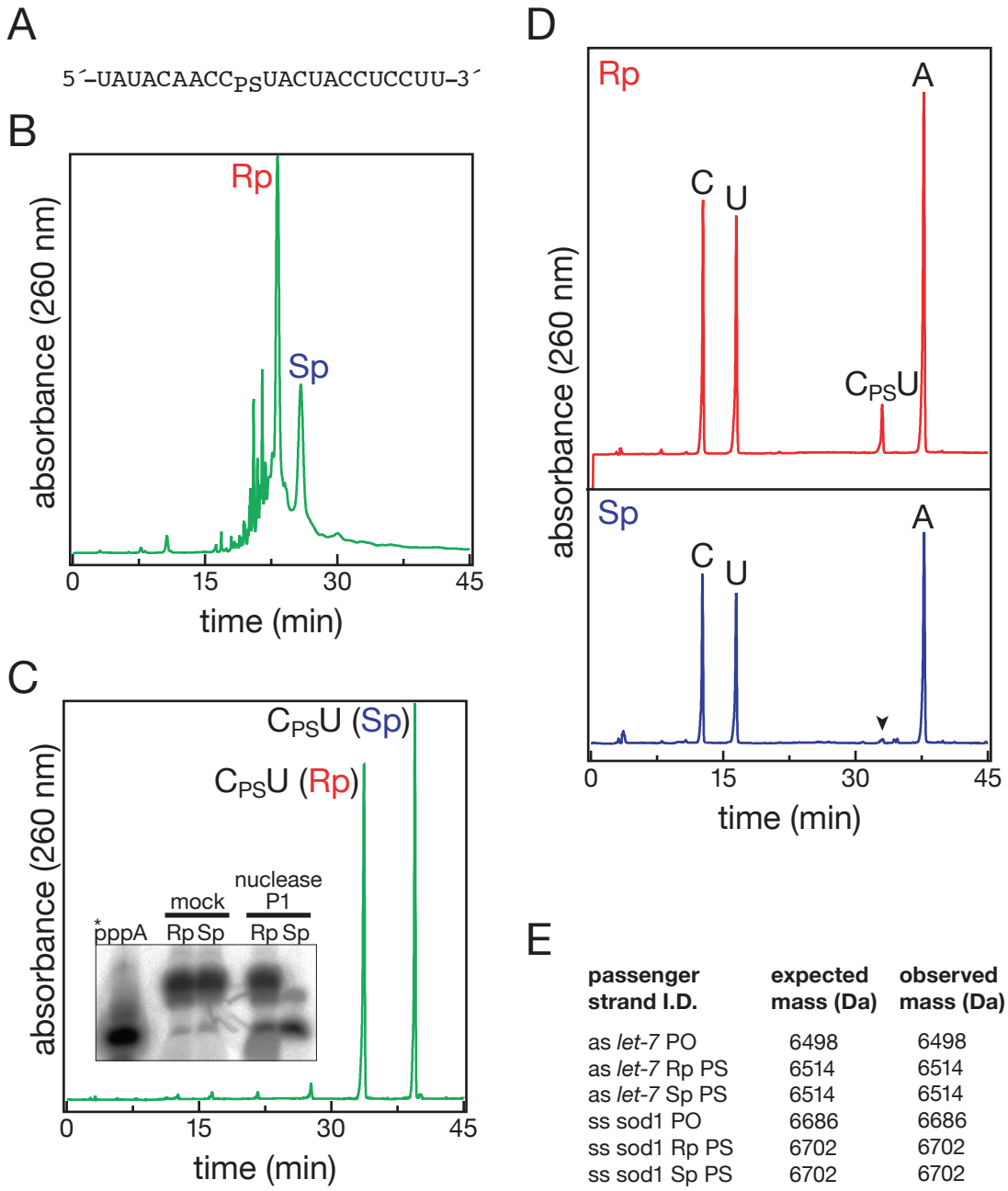


Figure Legend III-S3. A single, racemic phosphorothioate modification on the passenger strand inhibited siRNA unwinding. Production of single-stranded guide siRNA, a measure of active RISC assembly, was monitored using a 5′-^{32} P-radiolabeled guide strand (red) annealed to a passenger containing a racemic phosphorothioate at the linkage between nucleotides 9 and 10 or between nucleotides 10 and 11 (A) or between nucleotides 8 and 9 of the passenger strand (B). Data in (B) correspond to the average of three trials ± standard deviation. The rates of unwinding of the siRNAs bearing racemic phosphorothioate modification between passenger strand nucleotides 10 and 11 or 8 and 9 were essentially indistinguishable from that of the all-phosphodiester siRNA (see Figure 5E).

Figure III-S4



E

passenger strand I.D.	expected mass (Da)	observed mass (Da)
as *let-7* PO	6498	6498
as *let-7* Rp PS	6514	6514
as *let-7* Sp PS	6514	6514
ss sod1 PO	6686	6686
ss sod1 Rp PS	6702	6702
ss sod1 Sp PS	6702	6702

Figure S4. Purification of the phosphorothioate-modified siRNA diastereomers by reverse-phase, HPLC. (A) The sequence of the phosphorothioate (PS) -substituted *let-7* siRNA passenger strand. (B) The diastereomers of the passenger strand were resolved by reverse-phase, HPLC on a C18 column. The identity of the Rp and Sp peaks were confirmed by sensitivity to nuclease P1 (D) and by mass spectrometry (E). (C) Chiral dinucleotide standards were prepared by separating a racemic mixture of a PS-modified CPSU dinucleotide by reverse phase-HPLC. The identities of the two peaks were confirmed by their resistance (Rp) or sensitivity (Sp) to digestion with nuclease P1 (inset). (D) The HPLC-purified, 21-nt passenger-strand diastereomers were treated with nuclease P1, and then with calf intestinal phosphatase to remove 5´-monophosphates, and, lastly, resolved by reverse phase-HPLC of the resulting nucleosides. The Rp phosphorothioate dinucleotide prepared in (C) was used as a marker to confirm the identity of the CPSU peak in (D). The early eluting peak in (B) corresponds to the Rp diastereomer, because it contains a CPSU dinucleotide refractory to nuclease P1. The late eluting peak in (B) was fully converted to nucleosides by treatment with nuclease P1 followed by phosphatase and must therefore be the Sp diastereomer. A, adenosine; C, cytidine; U, uridine. Antisense *let-7* contains no guanosine. (E) The purified 21-nt RNA diastereomers were analyzed by negative ion electrospray mass spectrometry to confirm their integrity.

Figure III-S5

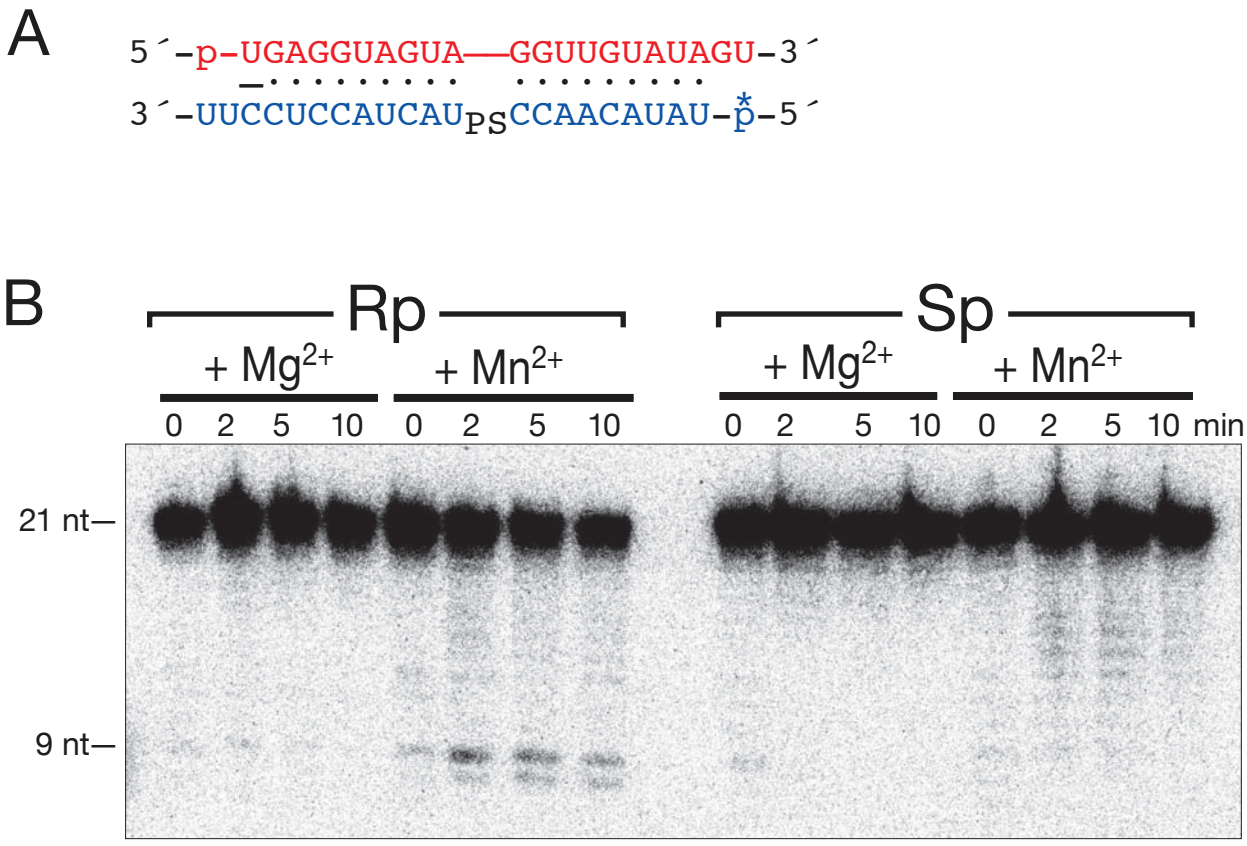


Figure S5. Phosphorothioate modification of the scissile passenger-strand phosphate inhibited cleavage for the *let-7* siRNA. (A) Diastereomers of the *let-7* siRNA passenger strand were purified for the *let-7* siRNA. (B) Passenger cleavage was detected for the Rp diastereomer only in the presence of supplemental Mn2+. No passenger-strand cleavage was observed for the Sp siRNA.

Figure III-S6

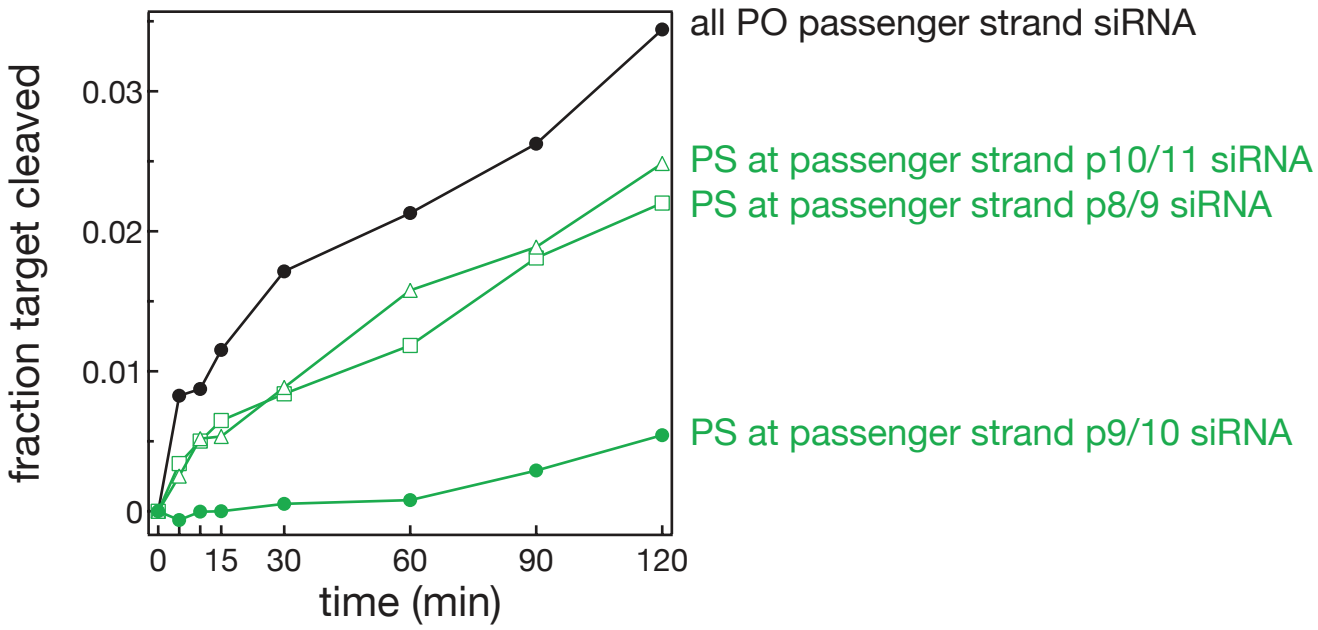


Figure S6. Phosphorothioate modification of the scissile phosphate of the passenger strand dramatically inhibited active RISC assembly in HeLa S100, relative to modification of the adjacent linkages. Target cleavage activity was monitored using sod1 siRNA with racemic phosphorothioate modification (PS) between nucleotides 8 and 9, 9 and 10 (the scissile phosphate), or 10 and 11 of the passenger strand. The sod1 siRNA with an all-phosphodiester (PO) passenger strand was used as a control.

CHAPTER IV: ATP requirements for assembly of Ago2 RISC

Summary

siRNAs are the specificity determinants of RNAi, a gene regulatory mechanism generally leading to target mRNA destruction. Assembly of siRNA into effector complexes—RISCs—requires many components including the ATPase Armitage (Armi) and RNaseH-like Argonaute2 (Ago2). In fungi and in animals, Argonaute cleaves the siRNA strand excluded from RISC, known as the passenger strand, facilitating assembly of mature single-stranded effector. Here we show assembly of an intermediate complex—pre-RISC—that contains at least Ago2 and duplex siRNA. Moreover, we dissect the ATP requirements for the transition of pre-RISC to mature single-stranded RISC using novel methods.

Results and Discussion

Passenger-Strand Cleavage Requires ATP

Assembly of Ago2-RISC from siRNA requires ATP (Nykanen et al. 2001; Haley et al. 2003; Schwarz et al. 2002; Tomari et al. 2004a). We wanted to determine if cleavage of the passenger strand has an ATP-requirement. To test if the passenger is cleaved in the absence of high levels of ATP—1 mM under conditions in our RNAi assay—0-2 hr *Drosophila* embryo lysate was dialyzed to deplete ATP from the lysate. By this method, we could deplete ATP to a concentration 100,000 fold-less than that in our normal reactions. At approximately 10 nM ATP, we found passenger strand cleavage to be

inefficient (Figure IV-1A). Passenger strand cleavage is greatly enhanced by the addition of ATP, suggesting that an ATP-dependent cofactor facilitates loading of duplex siRNA into Ago2.

The cysteine-alkylating agent, *N*-ethylamaleimide (NEM) blocks RISC assembly, but not the activity of RISC, in fly embryo lysate (Nykanen et al. 2001; Tomari et al. 2004a). Is passenger strand cleavage sensitive to NEM? To test if passenger strand cleavage is sensitive to NEM, we measured passenger strand cleavage from an asymmetric siRNA assembled in NEM-treated lysates. We found that passenger strand cleavage is blocked by NEM (Figure IV-1B). However, when lysate was treated with the reducing agent, dithiothreitol (DTT), prior to NEM, passenger strand cleavage was unaffected suggesting that NEM blocks passenger strand cleavage, perhaps by preventing assembly of Ago2-duplex siRNA complexes.

The ATPase *Armitage* is required for passenger strand cleavage

Drosophila *armitage* (*armi*) is a putative ATPase/DExH helicase with significant homology to SDE3 and MOV10, two helicases implicated in RNA silencing in plants and mammals respectively (Cook et al. 2004; Tomari et al. 2004; Meister et al. 2005). Mutations in *armi* disrupt siRNA-guided target cleavage and assembly of RISC in ovary lysates (Tomari et al. 2004). However, RISC-loading complexes (RLCs) are formed when siRNAs are incubated in *armi* lysates (Tomari et al. 2004), suggesting that *armi* may be required for loading duplex siRNA into Ago2. To test this idea, we incubated siRNA which contained 5´ radiolabeled passenger strand in ovary lysates prepared from

Figure IV-1

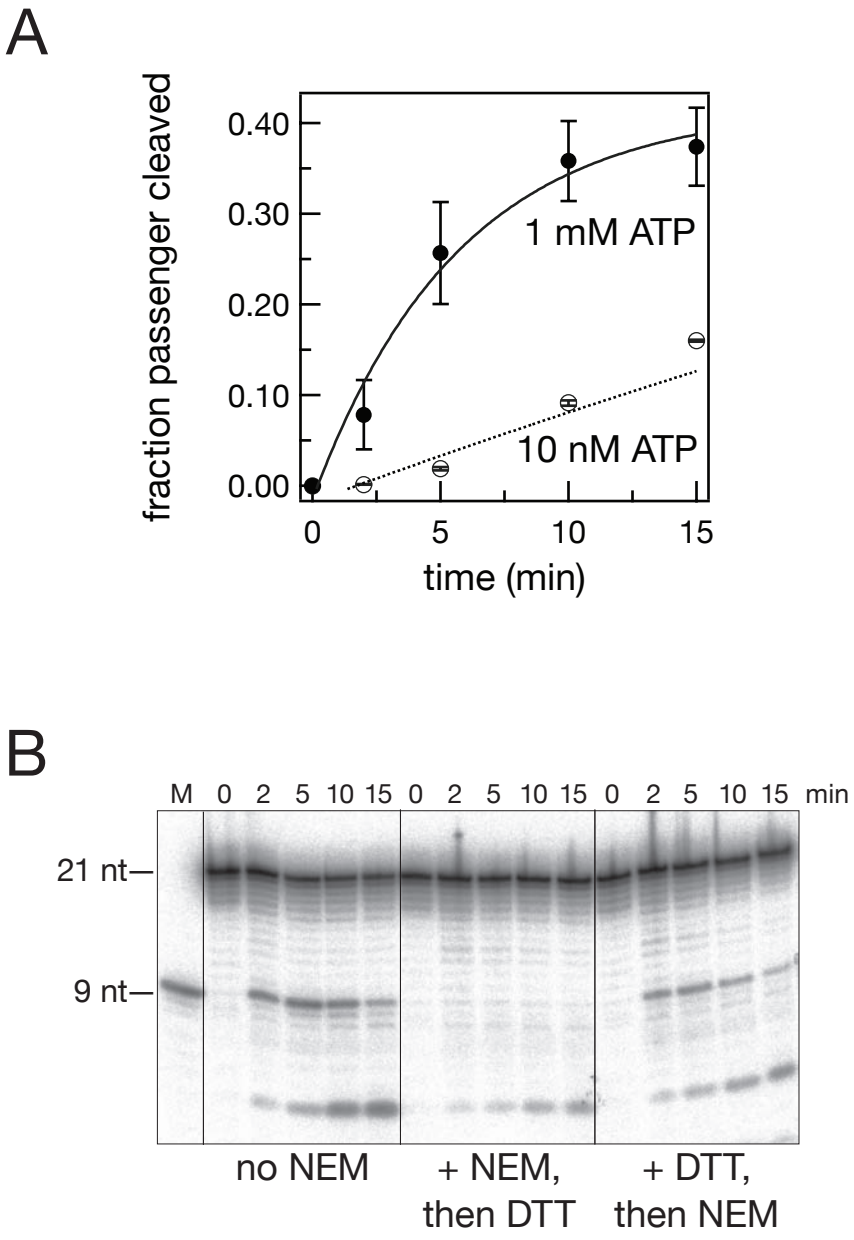

A
fraction passenger cleaved
0.40
0.30
0.20
0.10
0.00
1 mM ATP
10 nM ATP
0
5
10
15
time (min)
B
M 0 2 5 10 15 0 2 5 10 15 0 2 5 10 15 min
21 nt—
9 nt—
no NEM
+ NEM, then DTT
+ DTT, then NEM

Figure Legend IV-1. Passenger strand cleavage requires ATP and is sensitive to NEM. (A) The siRNA passenger strand is cleaved at 1 mM ATP; however, passenger strand cleavage is inefficient at concentration equal to that of the siRNA (10 nM). (B) NEM blocks passenger strand cleavage, most likely by preventing pre-RISC—Ago2 and duplex siRNA—assembly.

a strong *armi* mutant allele (*armi*$^{72.1}$) and measured cleavage of the passenger strand (Figure IV-2). There was no detectable passenger strand activity in *armi*$^{72.1}$ lysates, suggesting that *armi* is required for loading Ago2 with siRNA.

Assembly of pre-RISC with phosphorothioate-modified passenger strand

Previously, we found that phosphorothioate (PS) substitution of the scissile phosphate blocks cleavage of the passenger strand, just as it blocks target mRNA cleavage (Schwarz et. 2004; Chapter III). Moreover the Rp-PS diastereomer blocks cleavage of the passenger strand, and cleavage of this bond can be rescued by the addition of thiophilic Mn^{2+} (Chapter III). When using an siRNA that contains an Rp-PS diastereomer at the scissile phosphate, assembly of mature Ago2 RISC should be delayed in the absence of Mn^{2+}. Using Rp-PS modified siRNAs, can we assemble pre-RISC, a complex in which Ago2 is bound to duplex siRNA and cannot cleave the passenger strand (Kim et al. 2007)? To test if we can assemble Ago2 pre-RISC, we incubated an siRNA with an Rp-PS substitution between the 9th and 10th nucleotides of the passenger strand in 0-2 hr *Drosophila* embryo lysate for 10 min. After 10 min, NEM was added to the reaction to stop assembly and ATP was depleted by dialysis of the reaction. To determine of ATP is required for release of the passenger strand, we assayed loss of product—degradation of the 9 nt 5´ cleavage product—over time after adding back additional ATP. We assume that stable or nicked duplex siRNA protects the 9 nt 5´cleavage product from degradation. However, if the 9 nt is freed from guide strand and Ago2, then it would be susceptible to nucleases in the lysate. We found that after 10 minutes assembly, virtually

Figure IV-2

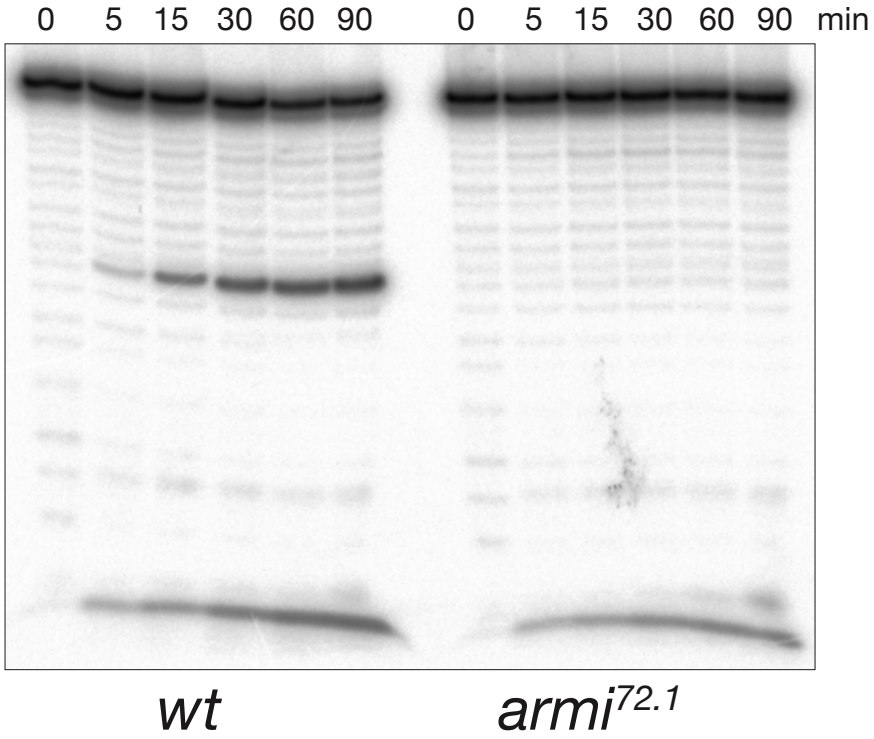


Figure IV-2. Armitage is required for passenger strand cleavage. Passenger strand radiolabeled siRNA was incubated in ovary lysate prepared from wildtype and armi (*armi*$^{72.1}$) mutants and cleavage of the passenger strand was monitored.

no passenger is cleaved from the Rp-PS passenger strand (before depletion). After ATP depletion, passenger strand cleavage was measured in the presence of Mn^{2+}. We found that upon addition of Mn^{2+}, a 9 nt cleavage product accumulates from the radiolabeled passenger strand (Figure IV-3). In the presence of 1 mM ATP, the cleavage product disappears with time, suggesting that the 9 nt product is released from the guide strand, and degraded in the lysate. Addition of a 2´-*O*-methyl oligonucleotide complementary to the passenger strand to the lysate stabilized the 9 nt 5´cleavage product in the presence and absence of ATP. Nuclease activity may require ATP; control experiments will be done to determine if ATP is necessary for degradation of the 9 nt 5´ cleavage product.

Purification of pre-RISC using Strep-tagII siRNAs

siRNAs can be synthesized of modifications as a tool to identify and purify siRNA-interacting proteins in our system. One method—the Strep-tagII technique—takes advantage of the biotin-streptavidin binding in which an eight residue peptide with characteristics of biotin binds an engineered version of streptavidin (known as streptactin) (Voss and Skerra 1997). Moreover, K_D of streptactin binding to the Strep-tagII peptide is in the micromolar range—a far less stable interaction than biotin-streptavidin. Thus, this interaction can be de-stabilized by free biotin, allowing for an elution step in the purification of siRNA bound material. siRNAs were synthesized with the Strep-tagII peptide, which was linked to the 3´ end of the guide strand (Figure IV-4A). To test if 3´ peptide-modified siRNAs are functional, we incubated the peptide-linked siRNA in 0-2 hr embryo lysate to assemble active RISC. After assembly

Figure IV-3

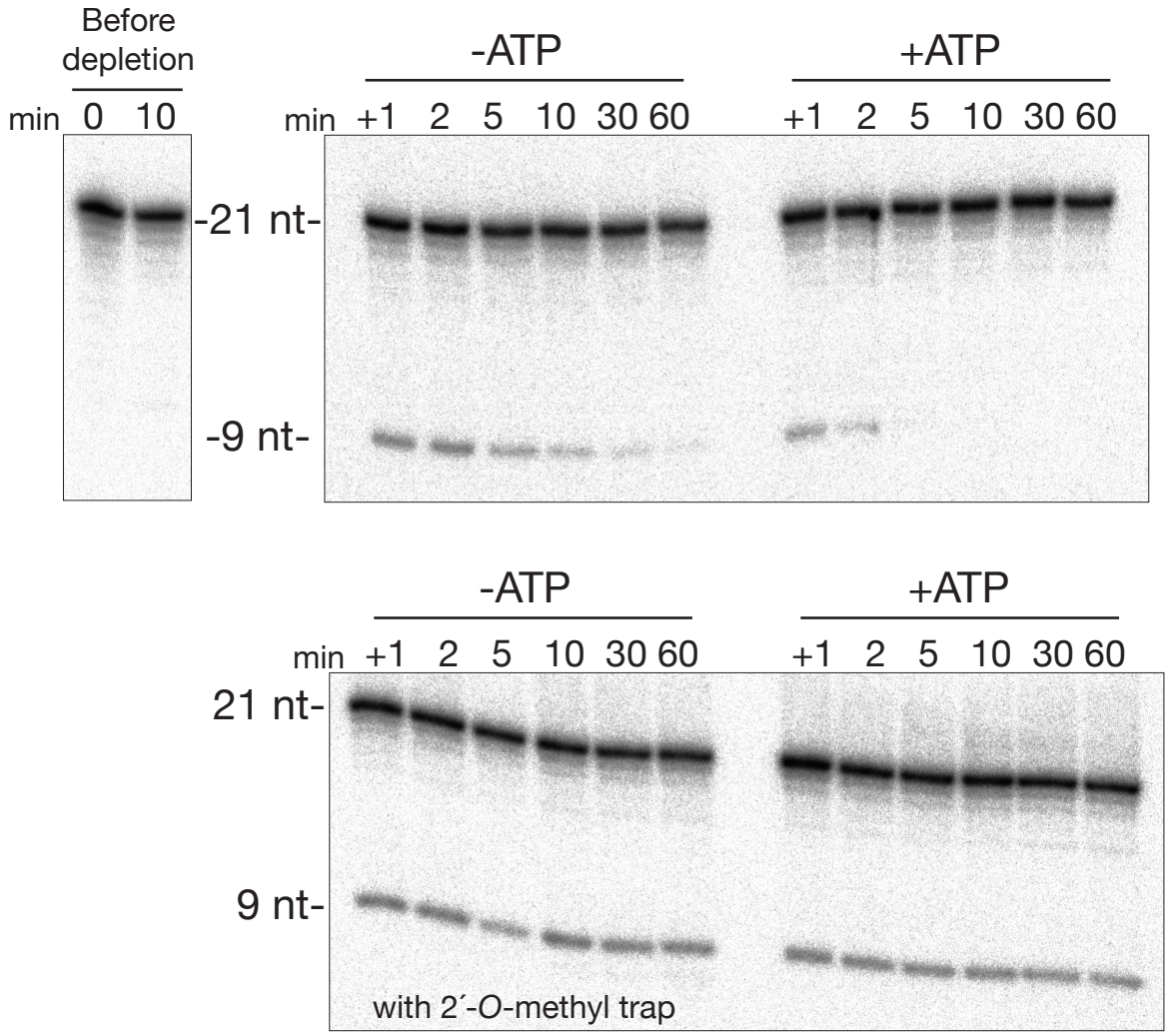


Figure Legend IV-3. Release of the cleaved passenger strand from the guide strand requires ATP. siRNA containing a radiolabeled Rp -PS passenger strand for 10 min in 0 - 2 hr embryo lysate for 10 min, and assembly was blocked with NEM and ATP was depleted from the reaction by dialysis. Release of the passenger strand from the guide strand was measured by loss of the 9 nt cleaved product over time. ATP is required for release of the cleaved product. Addition of a 2´-*O*-methyl oligo complementary to the passenger strand to the reaction prevented degradation of the 5´ 9 nt cleavage product.

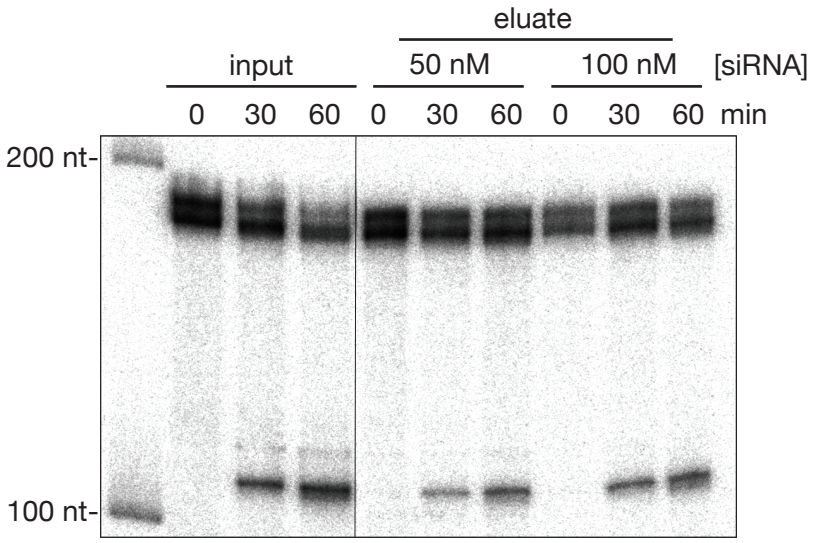
Figure IV-4

A

5´-UGAGGUAGUAGGUUGUAUAGU-p-3´-linker-S-Cys-Lys-Glu-Phe-Gln-Pro-His-Ser-Trp(NH_2)

3´-UUCCUCCAUCAUCCAACAUAU-5´

B



C

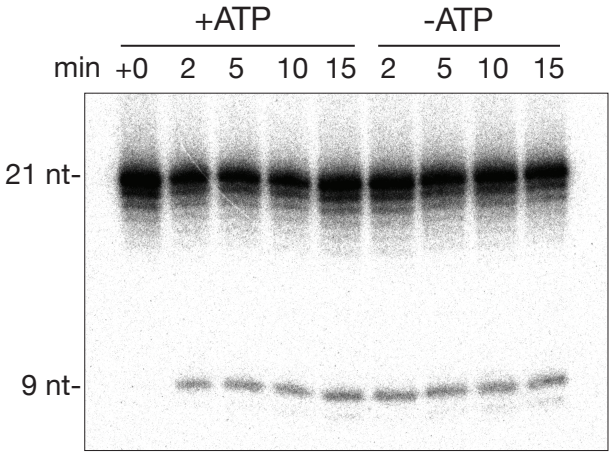


Figure Legend IV-4. Purification of pre-RISC complexes. (A) siRNA synthesized with an eight residue Strep-tagII peptide on the 3´end. (B) siRNAs with 3´ linked peptide retain the ability to assemble RISC in 0-2 hr embryo lysate. Cleavage activity of bono-fide cap radiolabeled target RNA in whole reactions (input) and eluate from streptactin batch purification. 50 nM and 100 nM Strep-tagII siRNAs were assembled in lysate, collected with streptactin beads, and eluted with biotin. Eluate was assayed for target cleavage activity. (C) pre-RISC captured under stringent conditions cleaves the passenger strand with or without ATP. Assembled and bound complex containing radiolabeled Rp-PS passenger strand and Strep-tagII guide strand was washed with 500 mM KOAc buffer and eluted from streptactin beads with desthiobiotin. 2.5 mM Mn^{2+} was added to the eluate and passenger strand cleavage was monitored +/- 1mM ATP.

with peptide-linked siRNAs, we assayed both whole reactions and eluate from streptactin resin for the ability to cleave target mRNA (Figure IV-4B). We found that peptide modified siRNAs were proper substrates for RISC assembly in whole lysate (input). Likewise, we found that eluted fractions contained target cleavage activity (eluate).

Can Ago2 pre-RISC be purified using the Strep-TagII method? To test this idea, we radiolabeled passenger strands containing an Rp-PS diastereomer in the scissile phosphate, then annealed these strands to 3´ Strep-tagII peptide-linked guide strands. We then incubated siRNAs in the lysate for 5 min (without Mn^{2+}), blocked assembly with NEM, captured siRNA-bound complexes with streptactin beads, washed bound portion in high salt buffer, eluted bound portion with desthiobiotin, and assayed the eluate for passenger cleavage (with Mn^{2+}). We found that the passenger strand is cleaved; and ATP does not effect cleavage or the stability of the cleaved product (Figure IV-4C).

Conclusions

We determined the ATP requirements for Ago2 passenger strand cleavage activity and pre-RISC formation in Drosophila embryo lysates. We found that passenger strand cleavage, like RISC assembly, is greatly enhanced by ATP (Nykanen et al. 2001; Tomari et al. 2004). Our data suggests that Ago2 pre-RISC comes in two forms: as part of a larger complex with other ATP-dependent cofactors (Figure IV-3); or in minimal form—Ago2 itself, duplex siRNA, and metal—captured by a Strep-tagII method (no ATP-cofactors) (Figure IV-4). Moreover, the ATPase containing, DExH/D box protein, *armi*, is required for passenger strand cleavage.

Why is ATP required for these steps of the RNAi pathway? Perhaps loading Ago2 with duplex siRNA requires some ATP-dependent conformational change in one or all of the components of the RLC and RISC. Interestingly, Dicer-2 and Armi have ATPase DExH motifs, suggesting that these proteins may link ATP hydrolysis with conformational change in protein or RNA structure. However, in Dicer-2, the DExH motif is required for siRNA production—not for RISC assembly by siRNA or siRNA unwinding—suggesting that this domain does not likely play a role in ATP-dependent RISC assembly (Lee et al. 2004; Tomari et al. 2004). Armi is required for passenger strand cleavage and RISC assembly suggesting its ATPase domain is functional during this process. It was postulated that a helicase is required for completely separating the strands of the siRNA during RISC assembly (Nykanen et al. 2001; Schwarz et a. 2003). Is Armi the siRNA unwindase? Armi may partially unwind the siRNA duplex, allowing Ago2 to bind an siRNA that resembles a more guide:target-like duplex (Parker et al. 2005). Alternatively, an ATP-dependent exonuclease may remove the passenger strand after pre-RISC formation. In Neurospora, QIP-1 exonuclease removes the nicked passenger duplex after cleavage by its Argonaute protein—QDE-2 (Maiti et al. 2007). This exonuclease function may be conserved in animals. Future genetic and biochemical assays will be done to determine the requirements for RISC assembly in Drosophila.

Materials and Methods

General Methods and Reagents

Preparation of 0–2 h embryo and ovary lysates and in vitro RISC assembly and RNAi reactions were as described (Haley et al., 2003). Strep-tagII peptide siRNAs were a kind gift from Dharmacon (Lafayette, CO).

Pre-RISC assembly

Pre-RISC was assembled using passenger strands which contain a single diastereomerically-pure Rp phosphorothioate (PS) at the scissile phosphate. Rp-PS passenger strands were 5´ ^{32}P-radiolabeled, and annealed to either the unmodified or Strep-tagII 3´ linked (Figure IV-3C) guide strands. After assembly of pre-RISC for 5 to 10 min, samples were treated with 10 mM NEM for 10 minutes on ice and quenched with 11 mM DTT. ATP was depleted from the fraction by dialysis, through three buffer changes (100 mM KOAc, 30 mM HEPES-KOH, 2 mM $Mg(CH_3CO_2)_2$, 15% glycerol) at 4° C. Strep-tagII siRNAs were bound by Streptactin magnetic beads (Qiagen) for 30 minutes on ice and washed 3 times with high salt buffer (500 mM KOAc, 30 mM HEPES-KOH, 10% NP-40). Bound fraction was eluted by addition of 1X desthiobiotin-containing Elution Buffer (IBA) on ice for 30 minutes. Eluate was collected and assayed for passenger cleavage activity as previously described (Chapter III).

CHAPTER V: The *Drosophila* RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC

Disclaimer: The following chapter was a collaborative effort. Experiments for Figures 2 (collaboration with CL), 4 (collaboration with MH), and 5 as well as Supplemental Figure 7 were done by the author. Experiments for the remaining figures were done by Chengjian Li (Figures 1, 2, 3, S1, S2), Michael Horwich (Figures 3, 4, S3, S6), Peng Wang (S4), Gwen Farley (S5), and Vasia Vagin (technical help).

Summary

In plants and animals, small silencing RNAs guide Argonaute proteins to repress gene expression by a set of related mechanisms collectively called RNA silencing pathways (Zamore and Haley, 2005; Meister and Tuschl, 2004). In the RNA interference (RNAi) pathway (Fire et al., 1998), small interfering RNAs (siRNAs) defend cells from invasion by foreign nucleic acids, such as those produced by viruses. In contrast, microRNAs (miRNAs) sculpt expression of endogenous mRNAs (Bartel and Chen, 2004). In animals, a third class of small RNAs, Piwi-interacting RNAs (piRNAs), defends the genome from molecular parasites such as transposons (Aravin et al., 2003; Aravin et al., 2007; Aravin et al., 2001; Vagin et al., 2006). Here, we report that piRNAs in flies contain a 2´-*O*-methyl group on their 3´ termini, a modification previously reported for miRNAs and siRNAs in plants (Yang et al., 2006) and piRNAs in mice (Kirino and Mourelatos, 2007; Ohara et al., 2007) and rats (Houwing et al., 2007). In plants, small RNA methylation is

catalyzed by the HEN1 protein and serves to protect miRNAs and siRNAs from degradation (Li et al., 2005; Yang et al., 2006; Yu et al., 2005). We find that the *Drosophila* homolog of HEN1, DmHen1, methylates the termini of both siRNAs and piRNAs. In the absence of DmHen1, both the length and abundance of piRNAs are decreased, and piRNA function is perturbed. Unlike plant HEN1, the *Drosophila* enzyme acts on single-stranded rather than duplex small RNAs, explaining how it can use as substrates both siRNAs—which derive from double-stranded precursors—and piRNAs—which do not (Houwing et al., 2007; Vagin et al., 2006). 2´-*O*-methylation of siRNAs may be the final step in assembly of the RNAi-enzyme complex, RISC, occurring after Argonaute is loaded with single-stranded RNA.

Results and Discussion

Drosophila piRNAs are 2´-*O*-methylated at their 3´ termini

In flies, both piRNAs (also known as repeat-associated siRNAs, rasiRNAs) and siRNAs, but not miRNAs, are modified at their 3´ termini (Pelisson et al., 2007; Vagin et al., 2006). We selectively labeled (Figure V-S1) the terminal nucleotide of *Drosophila melanogaster* 0–2 h embryo and mouse and bull testicular piRNAs. The resulting ^{32}P-radiolabeled nucleoside 2´ or 3´-monophosphates were resolved by two-dimensional thin-layer chromatography (2D TLC) using a solvent system that can resolve nucleoside 2´ monophosphates, nucleoside 3´ monophosphates, and 2´-*O*-methyl nucleoside 3´ monophosphates (Figure V-S2). Modified nucleoside monophosphates derived from the 3´ termini of piRNAs were identified by comparison to modified and unmodified

nucleoside 2´ and 3´ monophosphate standards (Figure V-1A). The terminal nucleotide of the piRNAs of all three animals co-migrated with 2´-*O*-methyl nucleoside 3´ monophosphate standards, but not with any unmodified nucleoside monophosphate standard. Since mouse piRNAs were previously shown to contain 2´-*O*-methyl modified 3´ termini using both mass spectrometry (Ohara et al., 2007) and a 2D TLC system (Kirino and Mourelatos, 2007) distinct from ours, we conclude that *Drosophila* and bull piRNAs also contain a 2´-*O*-methyl group at their 3´ termini.

Drosophila siRNAs are 2´-*O*-methylated at their 3´ termini

siRNAs, like piRNAs, are modified at their 3´termini (Pelisson et al., 2007; Vagin et al., 2006); do siRNAs have a 2´-O-methyl group at the 3´ end? To test if the ultimate nucleotide of siRNAs is 2´-*O*-methylated, we selectively labeled synthetic *Pp* luciferase (luc) siRNAs that were assembled in *Drosophila* 0-2 hour embryo lysate for 4 hours. The guide strand of the luc siRNA was 5´radiolabeled and contained an ultimate 3´ adenosine. After preparation of total RNA and extraction of the radiolabeled RNA from a polyacrylamide gel, the guide strand of the siRNA was subjected to our selective labeling procedure and subsequent TLC analysis (Figure V-2). In a mock experiment, total RNA was prepared from embryo lysate without exogenous siRNA**.** We extracted RNA that migrated to the same position as the radiolabeled guide strand of the luc siRNA in our polyacrylamide gel system. We found that the terminal nucleotide of the siRNAs co-migrated with 2´-*O*-methyl adenosine 3´ monophosphate standards, but not with any other modified or unmodified nucleoside monophosphate standard. The signal for 2´-*O*-

methyl adenosine 3´ monophosphate was significantly high above background found in the mock TLC. We conclude that, like piRNAs, siRNAs are modified with a 3´terminal 2´-*O*-methyl in *Drosophila*.

DmHen1 is required for piRNA modification in vivo

In *Arabidopsis*, the RNA methyltransferase, HEN1, modifies the terminal 2´ hydroxyl group of small silencing RNAs. In *Drosophila*, predicted gene CG12367, whose 1559 nucleotide mRNA encodes a 391 amino acid protein with a 220 amino acid evolutionarily conserved methyltransferase domain (Tkaczuk et al., 2006) most closely resembles *Arabidopsis* HEN1 (Figure V-1B) (Park et al., 2002). For simplicity, we call this gene *Drosophila melanogaster* (Dm) *hen1*. When homozygous, a piggyBac transposon insertion (PBac{WH}CG12367[f00810]) within the first intron of the fly *hen1* gene reduces the accumulation of *hen1* mRNA by 1,000-fold in testes and by more than 130,000-fold in ovaries (Figures V-3A and S3) and can therefore be considered a null mutation, which we refer to as $hen1^{f00810}$. We examined the 3´ termini of two types of highly abundant piRNAs in the germ line of flies heterozygous or homozygous for $hen1^{f00810}$. In testes, the *Suppressor of Stellate* (*Su(Ste)*) locus produces 24–27 nt rasiRNAs, a subclass of piRNAs that directs silencing of the selfish genetic element *Stellate*. *Su(Ste)* rasiRNAs, like other *Drosophila* piRNAs, are modified at their 3´ termini and therefore do not react with $NaIO_4$ (Vagin et al., 2006). In contrast, *Su(Ste)* rasiRNAs from $hen1^{f00810}/hen1^{f00810}$ mutant testes reacted with $NaIO_4$ and could therefore be β-eliminated to remove the last nucleotide of the RNA, increasing

Figure V-1

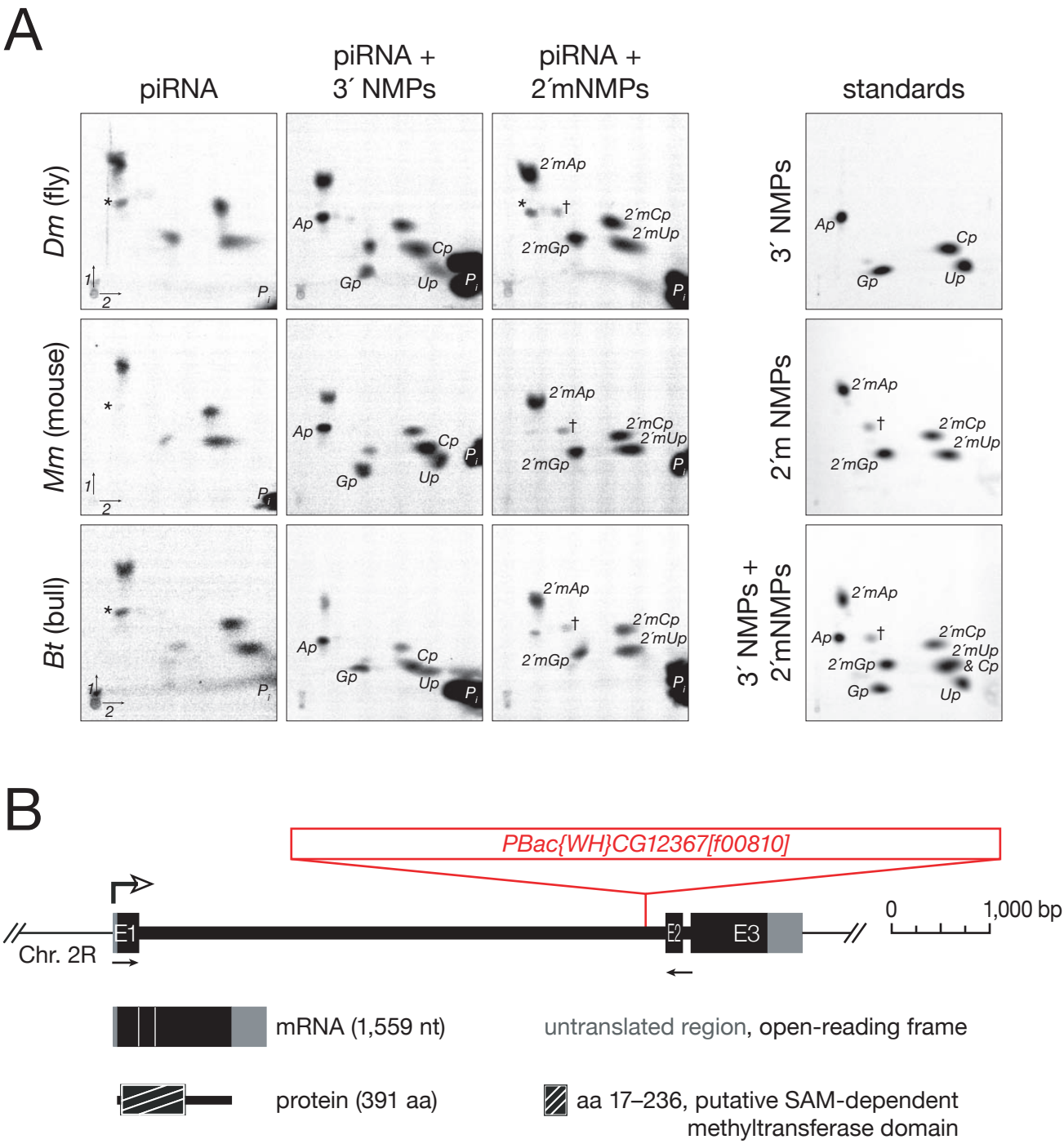

A
piRNA
piRNA + 3´ NMPs
piRNA + 2´mNMPs
standards
Dm (fly)
Mm (mouse)
Bt (bull)
3´ NMPs
2´m NMPs
3´ NMPs + 2´mNMPs
Ap
Cp
Gp
Up
Pi
2´mAp
2´mCp
2´mUp
2´mGp
& Cp
B
PBac{WH}CG12367[f00810]
Chr. 2R
E1
E2
E3
0
1,000 bp
mRNA (1,559 nt)
untranslated region, open-reading frame
protein (391 aa)
aa 17–236, putative SAM-dependent methyltransferase domain

Figure V-1. 2´-*O*-methylation of piRNAs in *Drosophila*. (A) Drosophila piRNAs are 2´-*O*-methylated at their 3´ termini. The modified nucleotides on the 3' termini of piRNAs from 0–2 h fly embryos, and mouse and bull testes were selectively ^{32}P radiolabeled. The radiolabeled modified mononucleotides from each species were resolved by 2D-TLC individually (piRNA), with ^{32}P-radiolabeled 3' mononucleotide standards (piRNA+3'NMPs), and with ^{32}P-radiolabeled 2'-*O*-methyl, 3' phosphate mononucleotide standards (piRNA+2'mNMPs). The modified nucleotides from the piRNA from all three animals co-migrated with 2'mNMPs standards, but not with 3'NMPs standards. Ap, 3'AMP; Gp, 3´GMP; Cp, 3´CMP; Up, 3'UMP; 2´mAp, 2'mAMP; 2´mGp, 2'mGMP; 2´mCp, 2'mCMP; 2´mUp, 2'mUMP; and P_i, phosphate. The asterisk marks a contaminant, likely 3' AMP, present in the [5´ ^{32}P] cytidine 5´,3´ bis-phosphate used to radiolabel the piRNA. † marks a contaminant present in the 2'-*O*-methyl, 3' phosphate mononucleotide standards. (B) *Drosophila hen1* gene (CG12367), mRNA, and protein. The piggyBac transposon, PBac{WH}CG12367[f00810], is inserted 207 bp upstream of the second exon. The open arrow indicates the predicted start of transcription. The closed arrows denote the position of the qRT-PCR primers used in Figure V-2A. The first intron of *hen1* contains another gene, CG8878, transcribed in the opposite direction, whose expression is unaltered by the piggyBac insertion (Figure V-S3).

their gel mobility (Figure V-2B) and indicating that in the absence of DmHen1 protein, they are not modified. Similarly, rasiRNAs that guide silencing of *roo*, the most abundant retrotransposon in *Drosophila melanogaster*, were not modified in *hen1*^{f00810} homozygous ovaries (Figure V-3C). The *Su(Ste)* and *roo* rasiRNAs were also shorter in the *hen1*^{f00810} homozygotes. In contrast, the length and amount of miR-8, which is expressed in both the male and female germ line, was unaltered in *hen1*^{f00810} homozygotes. For both *Su(Ste)* and *roo*, rasiRNAs were on average shorter and less modified even in *hen1*^{f00810} heterozygotes, compared to wild-type, suggesting that the abundance of DmHen1 protein limits the stability or production of piRNAs in flies.

DmHen1 is required for piRNA function in vivo

Modification of the termini of *Drosophila* piRNAs plays a role in their function: mRNA expression from *HeT*-A, the element whose expression is most sensitive to mutations that disrupt piRNA-directed silencing in the female germ line, more than quadrupled in *hen1*^{f00810} heterozygotes and was increased more than 15-fold in homozygotes, relative to wild-type tissue (Figure V-3D). We conclude that Hen1 protein is required for piRNA-directed silencing in the *Drosophila* germ line.

DmHen1 is required for siRNA modification

To test if DmHen1 is required for modification of the 3´ termini of siRNAs, we depleted Hen1 by RNAi in cultured *Drosophila* S2 cells. We transfected the cells with

Figure V-2

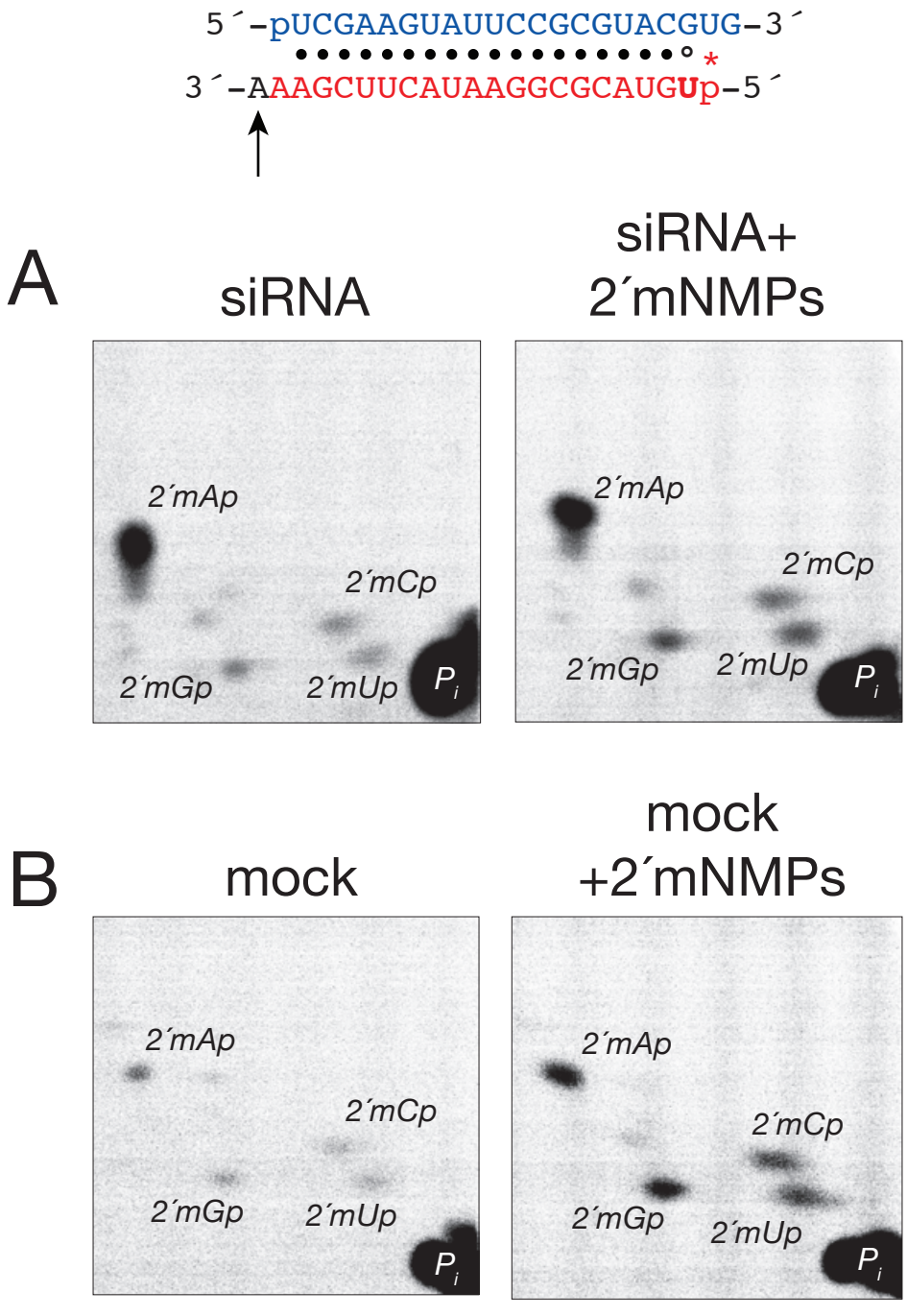

5´–pUCGAAGUAUUCCGCGUACGUG–3´
3´–AAAGCUUCAUAAGGCGCAUGUp–5´
A
siRNA
siRNA+
2´mNMPs
2´mAp
2´mCp
2´mGp
2´mUp
Pi
2´mAp
2´mCp
2´mGp
2´mUp
Pi
B
mock
mock
+2´mNMPs
2´mAp
2´mCp
2´mGp
2´mUp
Pi
2´mAp
2´mCp
2´mGp
2´mUp
Pi

Figure V-2. 2´-*O*-methylation of siRNAs in *Drosophila*. (A) Drosophila siRNAs are 2´-*O*-methylated at their 3´ termini. The modified nucleotides on the 3' termini of the guide strand of the *Pp* luciferase siRNAs assembled in 0–2 h fly embryo were selectively ^{32}P radiolabeled. The radiolabeled modified mononucleotides were resolved by 2D-TLC (siRNA), with ^{32}P-radiolabeled 2'-*O*-methyl, 3' phosphate mononucleotide standards (siRNA+2'mNMPs). The modified 3´ ultimate nucleotide from the siRNA—a 2´-*O*-methyl adenosine 3´ monophosphate—co-migrated with 2'mAMPs standard. (B) Total RNA from 0-2 hr embryo lysate was resolved on a polyacrylamide gel. RNA that co-migrated with the 21nt guide strand used in our experimental sample (in A) was collected, subjected to selective radiolabeling, and 2D-TLC.

long double-stranded RNA (dsRNA) targeting *hen1* on day 1 and day 5, then co-transfected them with both GFP (control) dsRNA and *hen1* dsRNA on day 8. Total RNA was harvested on day 9, probed for modification with $NaIO_4$/β-elimination, and analyzed by Northern hybridization using a 5´ ^{32}P-radiolabeled DNA probe complementary to the most abundant GFP-derived siRNA (MDH, Megha Ghildiyal, and PDZ, unpublished data). DsRNAs targeting two different regions of the fly *hen1* mRNA both reduced the amount of GFP siRNA modified at its 3´ terminus, whereas all the GFP siRNA remained modified when a control dsRNA was used (Figure V-3A).

Surprisingly, RNAi-mediated depletion of Ago2, but not Ago1, prevented the GFP siRNA from being modified. This result suggests that Ago2, but not Ago1, plays a role in the modification of siRNAs by DmHen1. To test this idea, we examined the modification status of the 3´ terminus of miR-277, an siRNA that partitions into both Ago1 and Ago2 complexes in vivo (Förstemann et al. 2007). *Drosophila* miRNAs associate predominantly or exclusively with Ago1 (Okamura et al., 2004) and have unmodified 3´ termini (Hutvágner et al., 2001; Pelisson et al., 2007; Vagin et al., 2006). In contrast, about half the miR-277 in cultured S2 cells failed to react with $NaIO_4$ (Figure V-3A), suggesting that about half of miR-277 is modified at its 3´ terminus. The fraction of miR-277 that was modified was reduced when two different dsRNAs were used to deplete DmHen1 by RNAi. When the cells were treated with dsRNA targeting *ago1*, all detectable miR-277 was modified, whereas all miR-277 became unmodified when dsRNA targeting *ago2* was used. In contrast, *bantam*, a miRNA that associates

Figure V-3

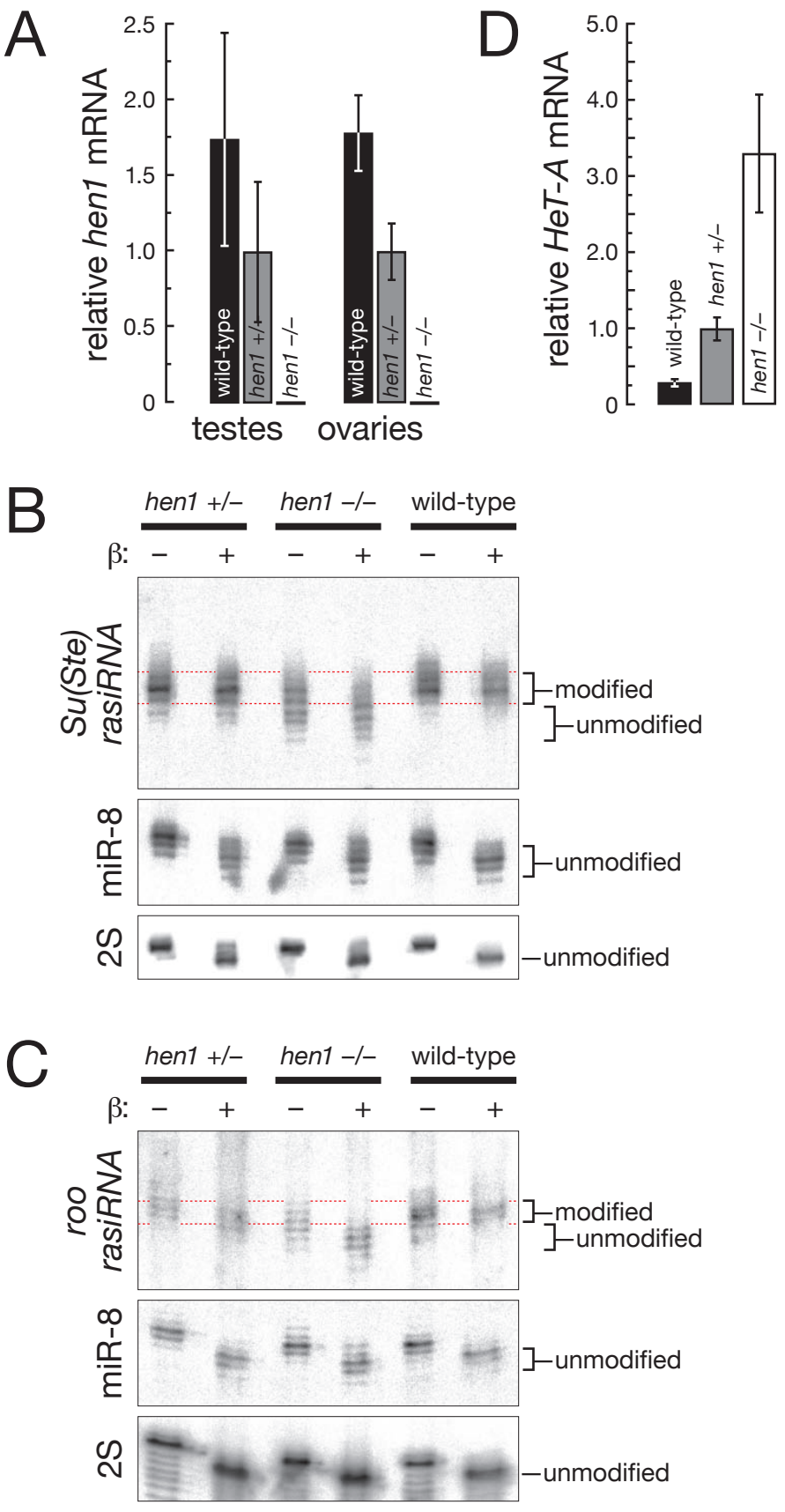

A
relative hen1 mRNA
2.5
2.0
1.5
1.0
0.5
0
wild-type
hen1 +/+
hen1 –/–
wild-type
hen1 +/–
hen1 –/–
testes
ovaries
D
relative HeT-A mRNA
5.0
4.0
3.0
2.0
1.0
0
wild-type
hen1 +/–
hen1 –/–
B
hen1 +/–
hen1 –/–
wild-type
β: – + – + – +
Su(Ste) rasiRNA
modified
unmodified
miR-8
unmodified
2S
unmodified
C
hen1 +/–
hen1 –/–
wild-type
β: – + – + – +
roo rasiRNA
modified
unmodified
miR-8
unmodified
2S
unmodified

Figure V-3. DmHen1 is required for normal piRNA biogenesis and complete silencing of the *HeT-A* Transposon. (A) Quantitative RT-PCR of *hen1* mRNA in testes and ovaries from wild-type or $hen1^{f00810}$ flies, relative to RP49. Bars report the average ± standard deviation for four or five independent experiments. (B, C) Northern hybridization to detect modification of small RNAs in wild-type and $hen1^{f00810}$ testes (B) or ovaries (C). The same blot was probed sequentially to detect the most abundant *Su(Ste)* rasiRNA (B) or the three most abundant *roo* rasiRNAs (C), miR-8, and 2S ribosomal RNA. (D) Quantitative RT-PCR of *HeT-A* transposon mRNA in ovaries. *HeT-A* mRNA levels were measured relative to RP49 using total RNA prepared from wild-type, $hen1^{f00810}$ heterozygous or homozygous ovaries. Bars report the average ± standard deviation for four or five independently prepared samples.

nearly exclusively with Ago1 (Okamura et al., 2004), was unmodified under all conditions (Figure V-4A).

siRNA modification correlates with Ago2-RISC assembly in vitro

siRNA modification can be recapitulated in lysates of either embryos or cultured S2 cells. Modification of siRNA in vitro was inhibited by *S*-adenosyl homocysteine, but not by *S*-adenosyl methionine, consistent with DmHen1 transferring a methyl group from *S*-adenosyl methionine to the terminal 2´ hydroxyl group of the RNA, thereby generating *S*-adenosyl homocysteine as a product (Figure V-S4). Our data from cultured S2 cells suggested that DmHen1 modifies that portion of miR-277 that enters the Ago2-RISC assembly pathway, but not the population of miR-277 that assembles into Ago1-RISC. To further test the idea that small RNA modification requires both Hen1 and the Ago2-RISC assembly pathway, we prepared cytoplasmic lysates from dsRNA-treated cultured S2 cells. Lysate from control-treated cells modified the 3´ terminus of a 5´ ^{32}P-radiolabeled synthetic siRNA duplex, but not lysate from *hen1*-depleted cells (Figure V-3B). The addition of either of two different preparations of purified, recombinant DmHen1, expressed in *E. coli* as a 74 kDa glutathione S-transferase fusion protein (GST-DmHen1; Figure V-S5), restored the ability of the lysates to modify the siRNA, indicating that loss of DmHen1 directly caused the loss of siRNA modification. Moreover, lysates depleted for Ago2, but not Ago1, could not modify the ^{32}P-siRNA in vitro (Figure V-3B). These in vitro data, together with our S2 cell experiments, suggest

Figure V-4

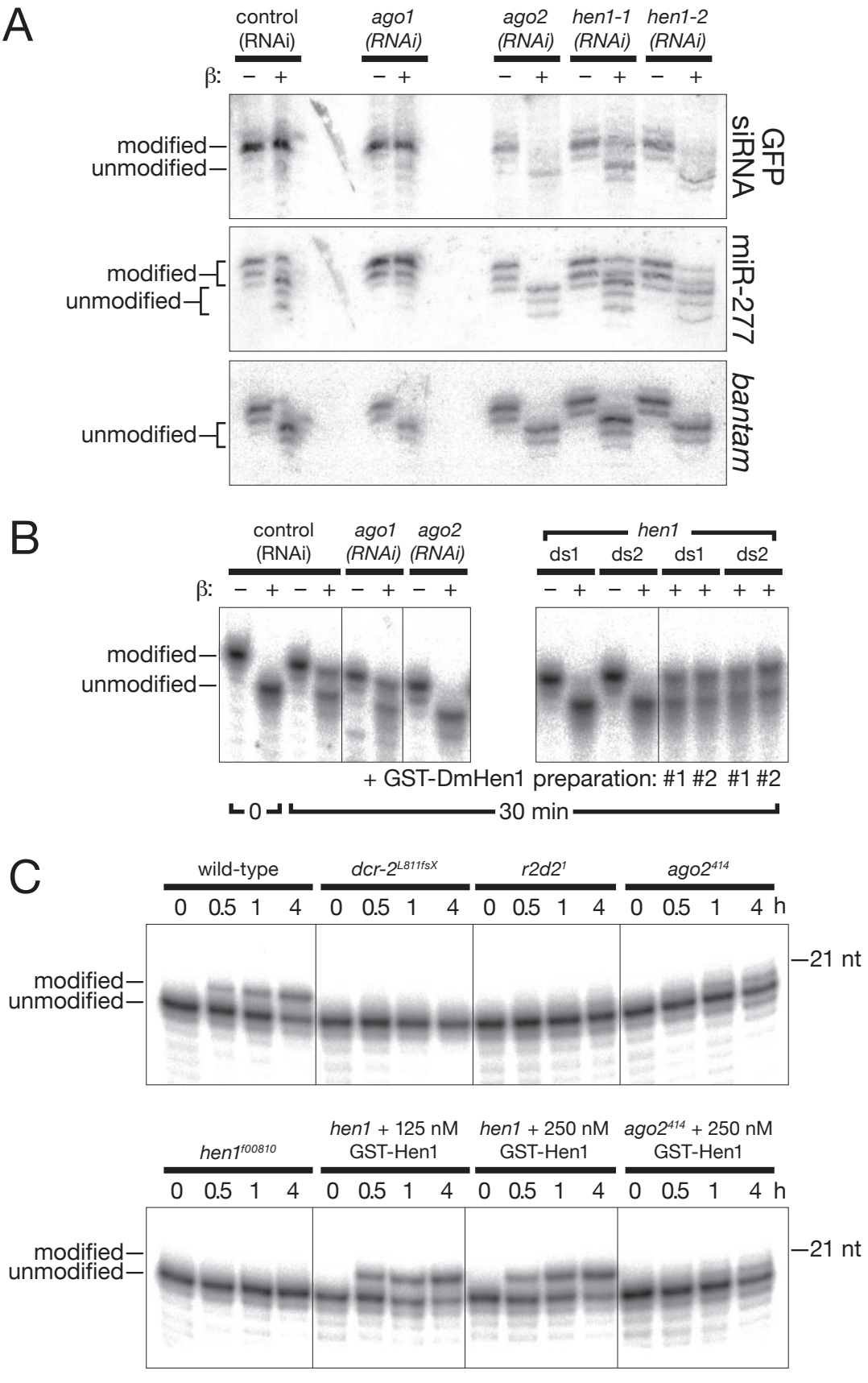

A
control (RNAi)
ago1 (RNAi)
ago2 (RNAi)
hen1-1 (RNAi)
hen1-2 (RNAi)
β: − + − + − + − + − +
modified
unmodified
GFP siRNA
modified
unmodified
miR-277
unmodified
bantam
B
control (RNAi)
ago1 (RNAi)
ago2 (RNAi)
hen1
ds1 ds2 ds1 ds2
β: − + − + − + − + − + + + + +
modified
unmodified
+ GST-DmHen1 preparation: #1 #2 #1 #2
0
30 min
C
wild-type
dcr-2L811fsX
r2d21
ago2414
0 0.5 1 4 0 0.5 1 4 0 0.5 1 4 0 0.5 1 4 h
modified
unmodified
21 nt
hen1f00810
hen1 + 125 nM GST-Hen1
hen1 + 250 nM GST-Hen1
ago2414 + 250 nM GST-Hen1
0 0.5 1 4 0 0.5 1 4 0 0.5 1 4 0 0.5 1 4 h
modified
unmodified
21 nt

Figure V-4. DmHen1 modifies Ago2-associated small RNAs. (A) Modification of siRNAs and miRNAs in *Drosophila* S2 Cells. A stable S2 cell line expressing GFP was treated with the indicated dsRNA alone (day 1 and 5), then together with GFP dsRNA (day 8). Two non-overlapping dsRNAs were used to target *hen1*. Total RNA was collected on day 9, treated with $NaIO_4$/β elimination, then dsGFP-derived siRNA, miR-277 and *bantam* detected by sequential Northern hybridization of the same blot. (B) In vitro siRNA modification in dsRNA-treated S2 cell lysates. (C) In vitro siRNA modification in *Drosophila* mutant ovary lysates.

that modification of the 3´ terminus of siRNAs and miRNAs is coupled to assembly into Ago2-RISC.

Dcr-2 and R2D2 act to load double-stranded siRNAs into Ago2. We prepared lysates from ovaries homozygous mutant for *hen1*, *dcr-2*, *r2d2*, and *ago2* using alleles unable to produce the corresponding protein (Lee et al., 2004; Liu et al., 2003; Okamura et al., 2004). A 5´ ^{32}P-radiolabeled siRNA duplex was incubated in each lysate to assemble RISC. At each time point, we determined if the siRNA was 3´ terminally modified by assessing its reactivity with $NaIO_4$ (Figure V-3C). No modified siRNA accumulated when the duplex was incubated in $hen1^{f00810}$, $dcr\text{-}2^{L811fsX}$, $r2d2^{1}$, or $ago2^{414}$ mutant lysate. Adding 250 nM purified, recombinant GST-DmHen1 restored siRNA modification to the $hen1^{f00810}$ but not the $ago2^{414}$ lysate. We conclude that the defect in $ago2^{414}$ reflects a requirement for Ago2 in small RNA modification by DmHen1, rather than an indirect effect, such as destabilization of DmHen1 in the absence of Ago2. GST-DmHen1 similarly rescued lysate from *hen1(RNAi)* but not *ago2(RNAi)* treated S2 cells (Figure V-S6). Together, the results of our experiments using cultured S2 cells—a somatic cell line—and ovaries, which comprise mainly germ line tissue, suggest that a functional Ago2-RISC assembly pathway is required for siRNA modification in *Drosophila*.

siRNAs are modified only after Ago2-RISC maturation

To test at which step in the Ago2-RISC assembly pathway siRNAs become modified, we determined if siRNAs are 2´-*O*-methylated by DmHen1 as single-strands or

as duplexes. In vitro, assembly of siRNAs into Ago2-RISC follows an ordered pathway in which the siRNA duplex first binds the Dicer-2/R2D2 heterodimer to form the RISC-loading complex (RLC). The RLC determines which of the two siRNA strands will become the guide for Ago2 and which will be destroyed (the passenger strand). The siRNA is then loaded into Ago2 as a duplex, with the passenger strand occupying the same position as future target RNAs, generating pre-RISC (Kim et al., 2006). Cleavage of the passenger strand by the Ago2 endonuclease domain converts pre-RISC to mature RISC (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005; Miyoshi et al., 2005). No single-stranded guide or passenger RNA is produced prior to this maturation step. Thus, all single-stranded siRNA produced in vitro or in vivo (Kim et al., 2006) corresponds to mature RISC.

We assembled Ago2-RISC in vitro using an siRNA designed to load only one of its two strands into Ago2 (Schwarz et al., 2003). We then sampled the reaction over time, isolating the 5´ ^{32}P-radiolabeled siRNA under conditions previously demonstrated to preserve its structure (Nykanen et al., 2001), and separated single- from double-stranded siRNA by native gel electrophoresis (Figure V-S7). Full-length siRNA duplexes and siRNA heteroduplexes comprising a full-length guide paired to a cleaved passenger strand co-migrate as double-stranded siRNA in these gel conditions (Matranga et al., 2005). The RNAs were then isolated from the gel and tested for reactivity with $NaIO_4$ to determine the presence of modification at their 3´ termini (Figure V-5A and B). At each time, total siRNA was analyzed in parallel. 3´ terminal modification increased over the course of RISC assembly and, at all times, was restricted to single-stranded siRNA:

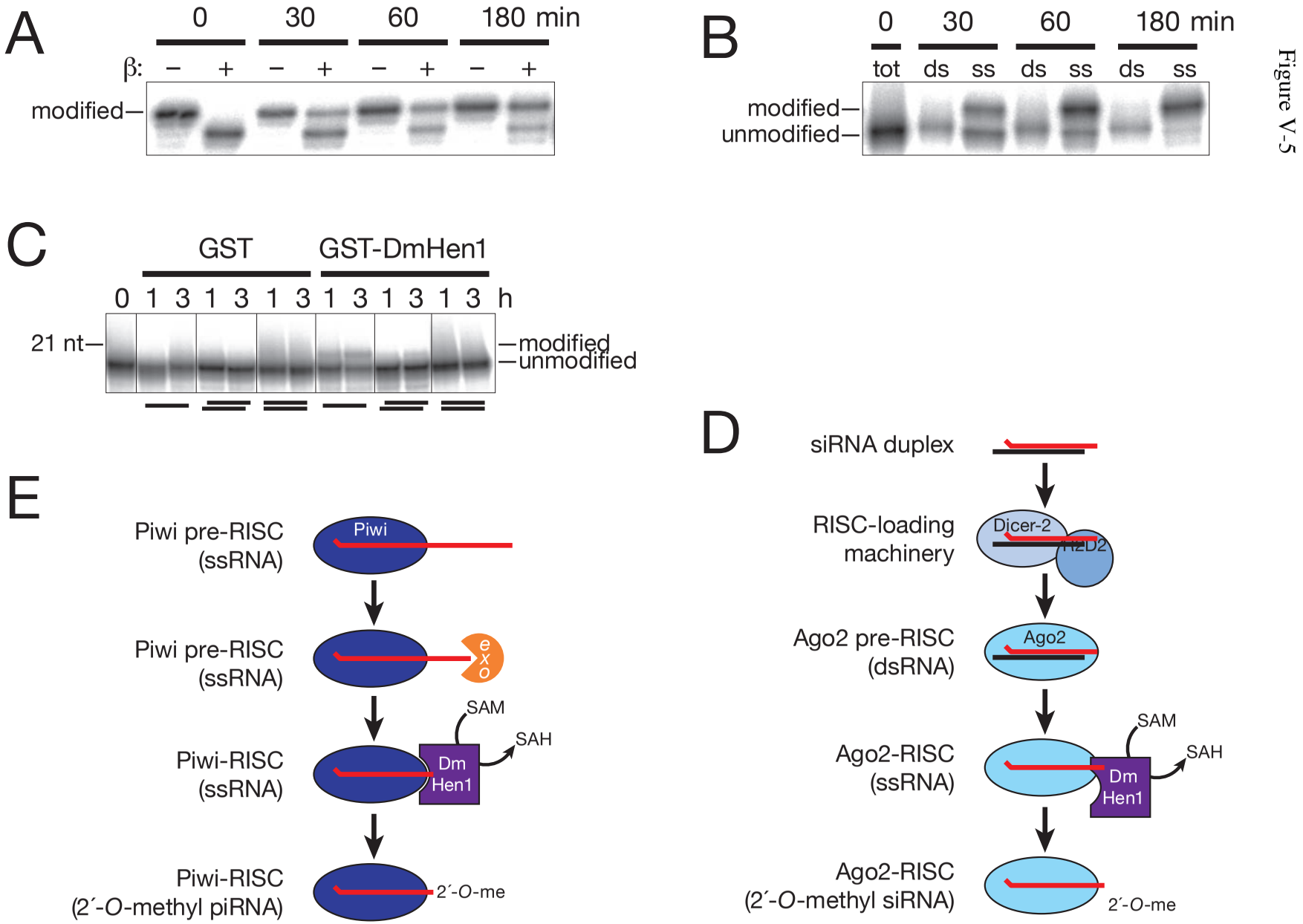

A
0
30
60
180 min
β: – + – + – + – +
modified—
B
0
30
60
180 min
tot ds ss ds ss ds ss
modified—
unmodified—
C
GST
GST-DmHen1
0 1 3 1 3 1 3 1 3 1 3 1 3 h
21 nt—
—modified
—unmodified
D
siRNA duplex
RISC-loading machinery
Dicer-2
R2D2
Ago2 pre-RISC (dsRNA)
Ago2
Ago2-RISC (ssRNA)
SAM
SAH
Dm Hen1
Ago2-RISC (2´-O-methyl siRNA)
2´-O-me
E
Piwi pre-RISC (ssRNA)
Piwi
Piwi pre-RISC (ssRNA)
exo
Piwi-RISC (ssRNA)
SAM
SAH
Dm Hen1
Piwi-RISC (2´-O-methyl piRNA)
2´-O-me

Figure V-5

Figure V-5. siRNAs are modified after the conversion of pre-RISC—which contains double-stranded siRNA—into mature RISC, which contains only single-stranded siRNA. siRNA duplexes with a 5´ ^{32}P-radiolabeled guide strand were incubated in *Drosophila* embryo lysate, and then tested for the presence of a 3´ terminal modification. (A) Total RNA from each time point in RISC assembly, without (–) and with (+) reaction with $NaIO_4$ and b-elimination. (B) Single- and double-stranded siRNA were resolved and purified from each time point in (A), then analyzed separately for the presence of a 3´ terminal modification on the siRNA guide strand. (A) and (B) are the left and right halves of a single gel. (C) Recombinant, purified GST-DmHen1, but not GST alone, can modify single-stranded 21 nt RNA, but not double-stranded siRNAs or blunt 21 nt RNA duplexes. However, in contrast to GST-DmHen1 incubated with *hen1*^{f00810} mutant ovary lysate (Figure V-3C), the enzyme alone is inefficient. (D) A proposed role for 2´-*O*-methylation in piRNA biogenesis. (E) A model for 2´-*O*-methylation of siRNAs.

within our limits of detection, all double-stranded siRNA was unmodified, even after 3 h. We conclude that siRNA modification is coupled to RISC assembly and occurs only after the conversion of pre-RISC to mature RISC.

Recombinant DmHen1 modifies single-stranded small RNA

While *Arabidopsis* HEN1 contains an N-terminal double-stranded RNA-binding motif (Yu et al., 2005), DmHen1 does not. To test if DmHen1 modifies double-stranded small RNAs, we incubated purified, recombinant, GST-DmHen1 with either single-stranded or double-stranded siRNAs. We detected modification, evidenced by loss of reactivity with $NaIO_4$, only for the single-stranded RNA, suggesting that DmHen1 modifies single-stranded substrates, but not siRNAs or blunt RNA duplexes (Figure V-4C). A preference for single-stranded RNA would explain how DmHen1 could act on both siRNAs, which are born double-stranded, and piRNAs, which are not. We note that the purified, recombinant GST-DmHen1 protein was more than 50-fold less active on its own than when supplemented with ovary lysate from *hen1*^{f00810} homozygous flies. We speculate that the Ago2-RISC machinery is required for Hen1 function in flies, although we cannot yet exclude the possibility that the lysate contains a small molecule or a protein modifying enzyme (e.g. a kinase) required to activate Hen1.

Modification of single-stranded siRNAs—that is, those associated with Piwi family proteins (Figure V-4D) or loaded in fully mature Ago2-RISC, but not double-stranded siRNAs (Figure V-4E) might allow cells to distinguish siRNAs loaded successfully into functional complexes from those that fail to assemble. For example, if a

3´-to-5´ nuclease acts to degrade single-stranded siRNAs, 2´-*O*-methylation of single-stranded siRNAs in Ago2-RISC may protect them from destruction. Moreover, such a nuclease might trim the 3´ end of piRNAs. 2´-*O*-methylation of the piRNA 3´ terminus may occur only when the length of RNA extending beyond the Piwi family protein is short enough to permit the simultaneous binding of the final ribose sugar to the active site of DmHen1 and the interaction of DmHen1 with the Piwi protein itself (Figure V-4D). Modification of the terminus of the trimmed piRNA would then block further 3´-to-5´ trimming of the small RNA, generating its Piwi-, Aubergine-, or Ago3-specific length. Our observation that piRNAs are shorter in *hen1*^{f00810} mutants supports this model.

We note that all 2´-*O*-methyl modified small RNAs identified thus far are associated with RISC complexes that efficiently cleave their RNA targets—i.e., Ago1-associated plant miRNAs, animal piRNAs, and Ago2-associated siRNAs in flies—whereas *Drosophila* miRNAs are typically both unmodified and associated with Ago1-RISC, which does not catalyze mRNA target cleavage in vivo (Tomari et al., *Cell*, in press). We speculate that DmHen1 is recruited to RISC complexes containing single-stranded small silencing RNAs according to the identity of their Argonaute protein. This model predicts that DmHen1 will bind only to complexes containing fly Ago2 or the three fly Piwi proteins, Piwi, Aubergine, and Ago3, but not Ago1. Clearly, future experiments will need to test this hypothesis.

Experimental Procedures

General Methods

Preparation of 0–2 h embryo, ovary, and S2 cell lysates and in vitro RISC assembly and RNAi reactions, and Northern hybridization were as described (Haley et al., 2003; Forstemann et al., 2005; Vagin et al., 2006). Sequences of synthetic RNA and DNA oligonucleotides are available online (Table S1).

^{32}P-radiolabeled 3´ mononucleotide standards

Synthetic RNA oligonucleotides (Table S1) were radiolabeled a 20 μl reaction containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 10% (v/v) DMSO, 10 μg/ml BSA, 2 units/μl RNasin (Promega, Madison, WI), 1.5 μCi/μl [5´ ^{32}P] cytidine 5´,3´ bis-phosphate ([5´ ^{32}P]-pCp; Perkin-Elmer, Waltham, MA), 1 unit/μl T4 RNA Ligase 1 (New England Biolabs, Ipswich, MA) at 4°C, overnight. The radiolabeled small RNAs were purified from a 15% denaturing urea-polyacrylamide sequencing gel, and then digested with 1.5 U/μl micrococcal nuclease (Takara Mirus Bio, Madison, WI) in a 40μl reaction containing 20 mM Tris-HCl pH 8.0, 5 mM NaCl, 2.5 mM $CaCl_2$. 3´ ^{32}P-mononucleotides were further purified from a 22.5% denaturing urea-polyacrylamide sequencing gel.

2D-TLC

Small RNAs (21-29 nt, containing both modified piRNAs, and unmodified small RNAs) from 0–2 h wild-type (Oregon R) fly embryos and small RNAs (26-31 nt, containing mostly modified piRNAs) from mouse and bull testes were purified from a 10% denaturing urea-polyacrylamide gel stained with SYBR® Gold (Invitrogen). To test the siRNA modification, RISC was assembled with 10 nM *Pp* luciferase siRNA (10% of siRNA contain 5´-^{32}P-radiolabeled guide strand) for 240 minutes. Radiolabeled siRNA and total RNA corresponding to 21 nt long oligoribonucleotides in our gel system (mock) was extracted from a 10% denaturing urea-polyacrylamide gel. About 100 pmole purified small RNAs were radiolabeled as described above, in a 40 μl reaction using 3μCi/μl [5´32p]-pCp and 1 unit/μl T4 RNA Ligase 1, and then gel purified. The purified, ^{32}P-radiolabeled RNA was hydrolyzed in 200mM Na_2CO_3 at 100°C for 1h, then neutralized with an equal volume of 200 mM HCl, dephosphorylated with 0.5 units/μl calf intestinal alkaline phosphatase (New England Biolabs) in a 200 μl reaction containing 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol. Alkaline phosphatase was inactivated by extraction with phenol /chloroform, the aqueous phase was oxidized with 80 mM $NaIO_4$ in borax/boric acid buffer (60 mM borax, 60 mM boric acid, pH 8.6) at room temperature for 30 min, and then β-eliminated with 200 mM NaOH at 45°C for 90 min. 5 μl of this reaction was mixed with an equal volume of formamide loading buffer (98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue), and resolved on a 22.5% denaturing urea-polyacrylamide sequencing gel. Equal intensities of modified

mononucleotides were spotted on 20 x 20 cm PEI-cellulose F glass TLC plates (EMD Chemicals, Gibbstown, NJ) and separated first with isobutyric acid/25%ammonia/water (66:1:33, v:v:v) and then 0.1M sodium phosphate pH6.8/ammonium sulfate/1-propanol (100:60:2, v:w:v).

Analysis of RNA 3´ termini

RNA was incubated for 30 min at room temperature with 25 mM $NaIO_4$ in borax/boric acid buffer (60 mM borax, 60 mM boric acid, pH 8.6), then 45.2 mM (f.c.) NaOH added, and incubation continued for 90 min at 45°C (β-elimination). The reaction was stopped by the addition of 300 mM (f.c.) NaCl, 1 μg glycogen, and three volumes absolute ethanol. After 30 min on ice, the precipitated RNA was collected by centrifugation.

Recombinant *Drosophila* Hen1 Protein

DmHen1 coding sequence was amplified from *Drosophila* ovary cDNA and inserted into pEnt-D-Topo (Invitrogen, Carlsbad, CA). The entire *hen1* sequence was confirmed by sequencing. The entry plasmid was recombined with the N-terminal GST expression vector, pDest-15, using LR Clonase (Invitrogen). GST-Hen1 was expressed in BL21 Star DE3 cells (Invitrogen) grown at 37°C in LB broth containing 100 ug/ml ampicillin until to OD_{600} reached 0.50. The culture was then cooled to 25°C and 0.4 mM IPTG added to induce protein production. The culture was incubated at 25°C with vigorous shaking for three hours. The cells were harvested by centrifugation at 7,300 x g

for 20 min, washed with PBS, centrifuged again, and then the cell paste frozen in liquid nitrogen and stored at -80°C.

Hen1 fusion protein was purified using the GST Purification Kit (Clontech, Mountainview, CA). Cells were resuspended in 40 ml of Extraction/Loading buffer and lysed by sonication (duty 30% for 6 minutes; Branson Sonificator II, Danbury, CT), with cooling in an ice bath. Two ml of clarified lysate was added to the column resin, and the column inverted several times to disperse the resin. The resin was then allowed to pack, and the remaining 38 ml passed through the column by gravity flow. Subsequent steps were according to the manufacturer's directions.

Analysis of double- and single-stranded siRNA

Double and single-stranded, 5´ ^{32}P-radiolabeled siRNA guide strands (10 nM; Figure V-S7 and 4) were separated as described (Nykanen et al. 2001). Briefly, RISC assembly reactions were stopped with 2x Proteinase K buffer, 2 mg/ml Proteinase K, 1 μg glycogen, and 250 nM unlabeled siRNA guide strand to prevent reannealing. After incubation for 30 min at 25˚C, 3 volumes absolute ethanol were added, and the RNA precipitated for 30 min on ice. The precipitates were collected by centrifugation, washed with 80% (v/v) ethanol, then dissolved in 2 mM Tris-Cl (pH 7.5), 3% (w/v) Ficoll-400, 0.04% (w/v) bromophenol blue, 100 mM KOAc, 30 mM HEPES-KOH, 2 mM $Mg(CH_3CO_2)_2$, and resolved by electrophoresis through a 15% native polyacrylamide gel (19:1 acrylamide:bis; 89 mM Tris-Borate, pH 8.3, 2 mM Na-EDTA, 2.5 mM $Mg(CH_3CO_2)_2$). The region of the native gel corresponding to double-or single-stranded

siRNA was excised, and the RNA eluted overnight in 1 M NaCl. 1 μg glycogen and ethanol (60% final volume) was added to the eluate, the RNA collected using MegaClear filter cartridges (Ambion), eluted with H_2O, and then precipitated for 30 min on ice by adding 500 mM (f.c.) $NH_4CH_3CO_2$ and 2.5 volumes absolute ethanol. The precipitate was collected by centrifugation, washed with 80% (v/v) ethanol, and the samples reacted with $NaIO_4$ and subsequent β-elimination (see above). The precipitated RNA was dissolved in 98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, and then resolved on a 15% denaturing urea-polyacrylamide sequencing gel.

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Figure V-S1

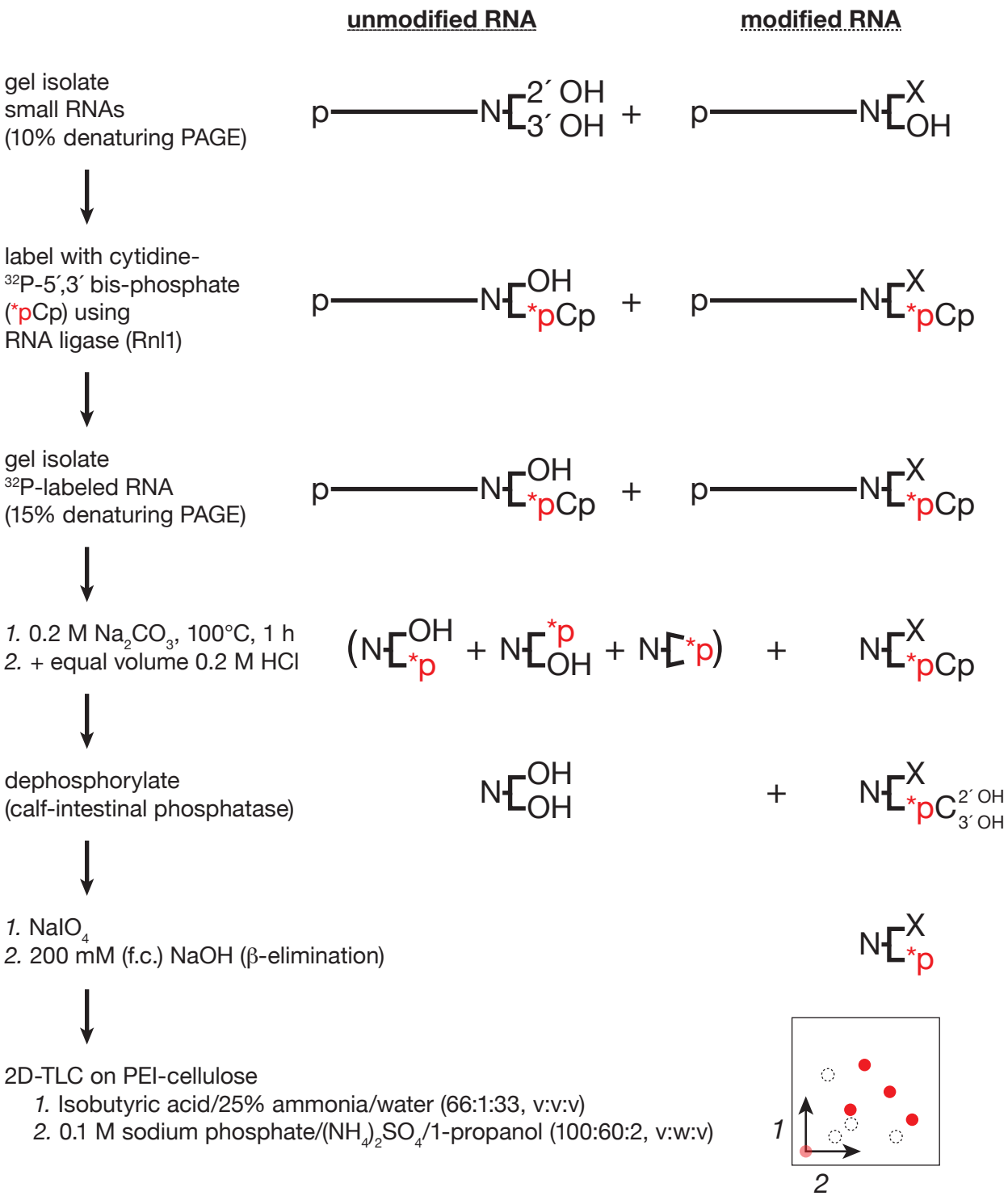

Figure V-S1. Scheme for selectively labeling the 3′ terminal nucleotide of modified small RNAs. Because Rnl1 will use either a 2′ or a 3′ hydroxyl as a ligation donor, the scheme will also label 3′ modified RNAs. Only phosphodiester linkages flanked by a 2′ hydroxyl are subject to base hydrolysis and only adjacent 2′ and 3′ hydroxyls react with $NaIO_4$, a prerequisite for β-elimination. X, 2′ or 3′ modification; *p, ^{32}P-radiolabeled phosphate group.

Figure V-S2

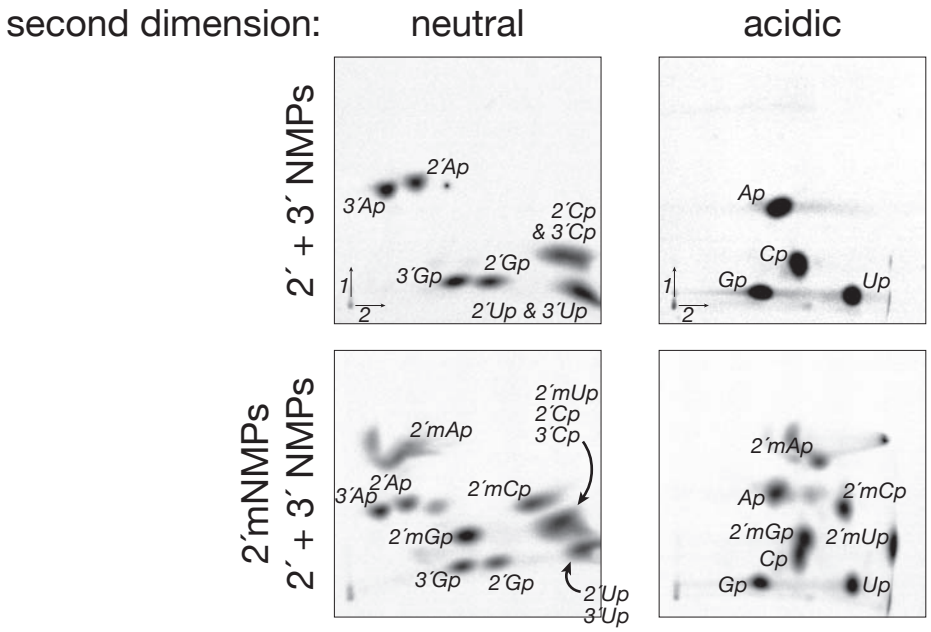

Figure S2. Comparison of 2D TLC systems. For both, the first dimension was Isobutyric acid/25% ammonia/water (66:1:33, v:v:v). Neutral second dimension (0.1 M sodium phosphate/$(NH_4)_2SO_4$/1-propanol [100:60:2, v:w:v]) this paper; acidic second dimension (2-propanol/HCl/H_2O (70:15:15, v:v:v), Kirino Y., and Mourelatos Z. (2007). Mouse Piwi-interacting RNAs are 2'-O-methylated at their 3' termini. Nat Struct Mol Biol. 14, 347-348. 2´ and 3´ NMPs were prepared by base hydrolysis, and 3´ NMP spots identified by their comigration with 3´ NMPs generated by complete digestion of RNA with micrococcal nuclease.

Figure V-S3

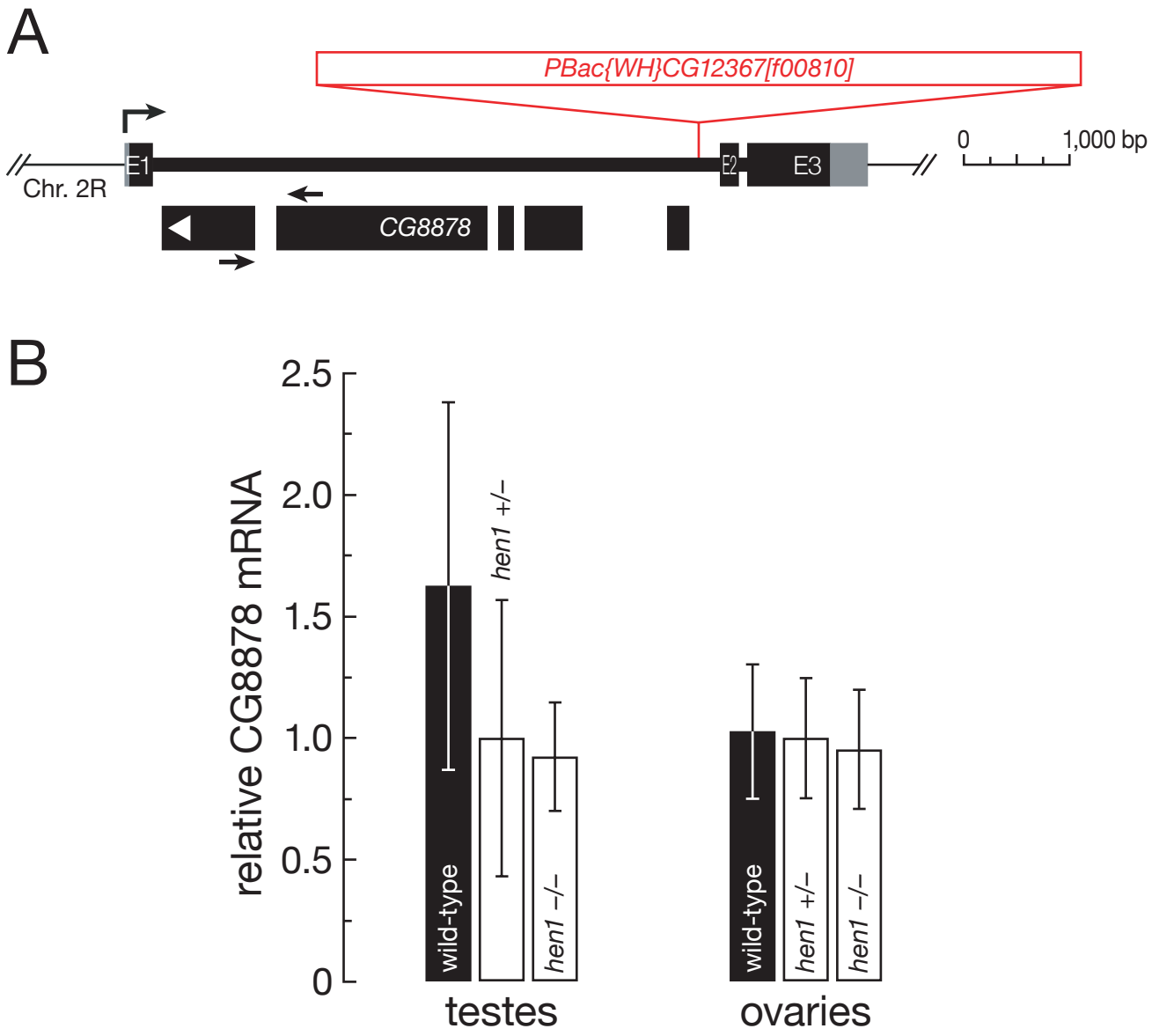

Figure S3. PBAC{WH}CG12367[f00810] disrupts *hen1* but not CG8878. (A) The gene CG8878 resides in the first intron of *Drosophila hen1* and is transcribed in the opposite orientation. The location of the qRT-PCR primers are shown as arrows. (B) We performed qRT-PCR to determine the effect on CG8878 expression of the PBAC{WH}CG12367[f00810] transposon insertion, which dirupts *hen1* (Figure 2A). In both testes and ovaries, we can detect no statistically meaningful effect of this piggyBac transposon insertion on the expression of CG8878. We conclude that the PBAC{WH}CG12367[f00810] insertion disrupts only *hen1*. We therefore rename this insertion $hen1^{f00810}$. Bars show average ± standard deviation for four or five independent trials.

FigureV-S4

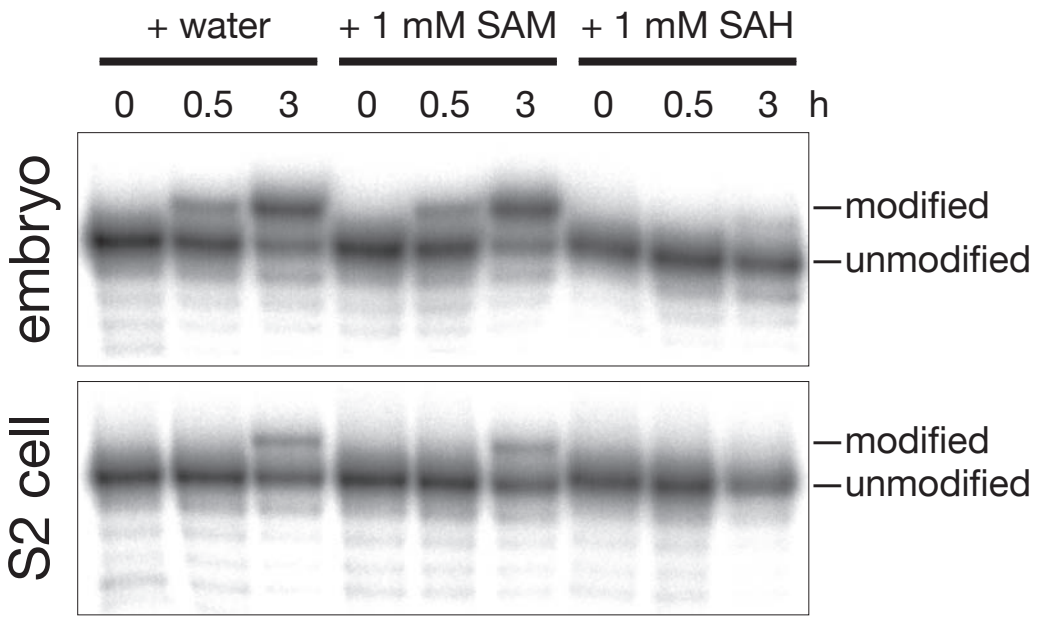

Figure S4. *S*-adenosyl homocysteine (SAH), an inhibitor of *S*-adenosyl methionine (SAM) dependent methyltransferases, inhibits modification of siRNA in lysate prepared from either 0–2 h embryos or cultured S2 cells. RNA was purified from the reactions at the indicated times and then tested for modification by reaction with $NaIO_4$ followed by β-elimination. The RNA was resolved by denaturing electrophoresis.

FigureV-S5

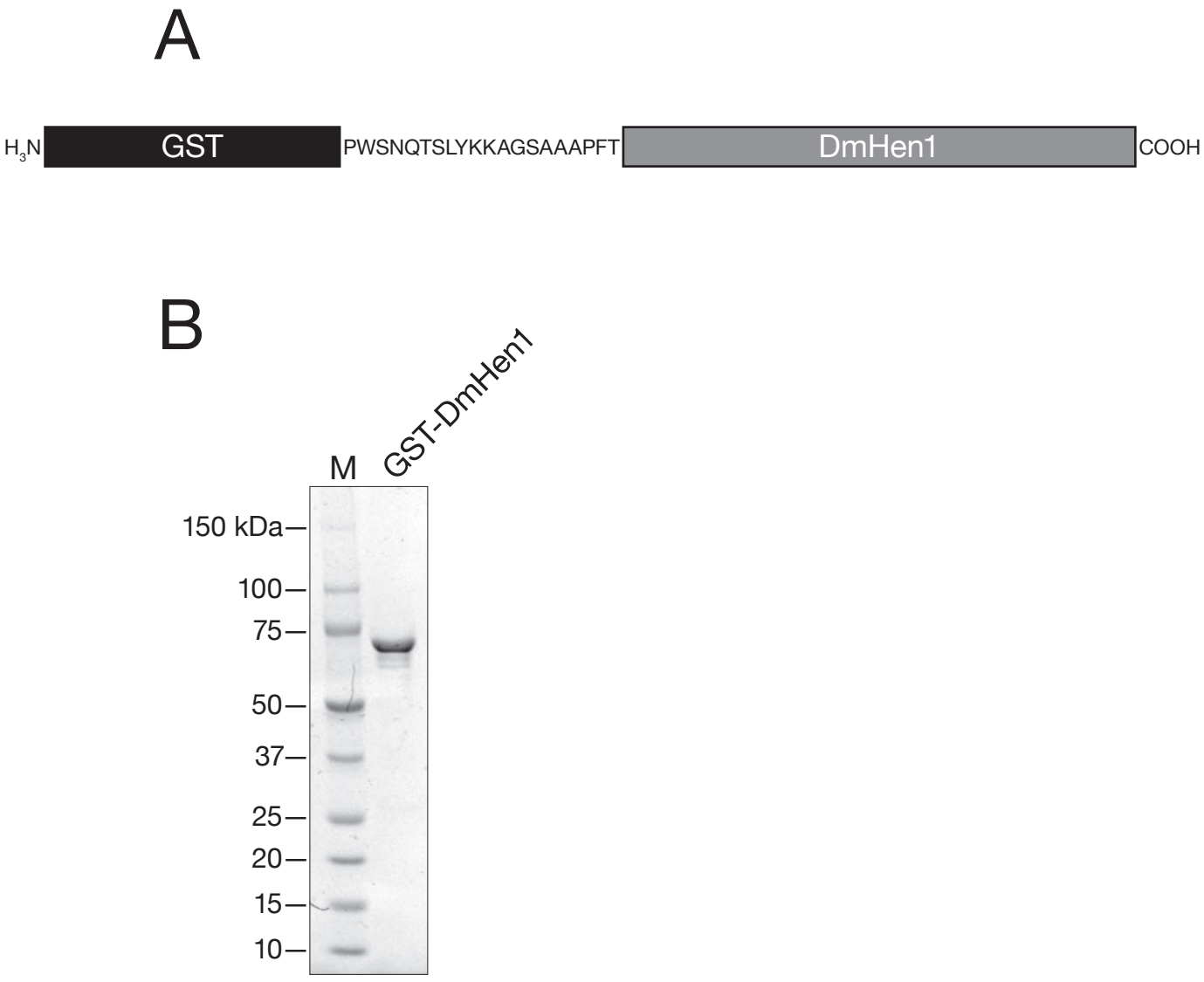

Figure S5. GST-DmHen1. (A) Schematic of the fusion protein, comprising the 225 amino acid glutathione S-transferase (GST) module, a 20 amino acid linker, and the entire 391 amino acid DmHen1 protein, terminated at its native stop codon. (B) Purified, recombinant GST-DmHen1 protein (3 μg) was resolved by electrophoresis through a 4–20% polyacrylamide gradient SDS-gel, then stained with colloidal Coomassie G-250. The apparent masses of molecular weight markers (M) are indicated.

FigureV-S6

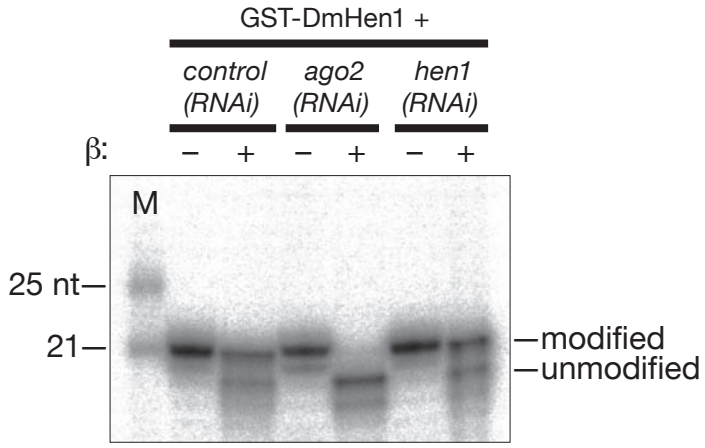

Figure S6. Purified, recombinant GST-DmHen1 protein rescues S2 cells depleted by RNAi of Hen1, but not Ago2. S2 cells were transfected as described with dsRNA targeting *ago2*, *hen1*, or a control sequence, then lysate prepared. siRNA was incubated in the S2 cell lysate to allow RISC assembly, then siRNA guide strand modification was assayed by reaction with $NaIO_4$ followed by β-elimination (β). Without the addition of GST-DmHen1 protein, *hen1(RNAi)* and *ago2(RNAi)* lysates are deficient in Hen1 activity, relative to the control dsRNA treated cells (Figure 3B). M, 5′-phosphorylated synthetic RNA size markers.

Figure V-S7

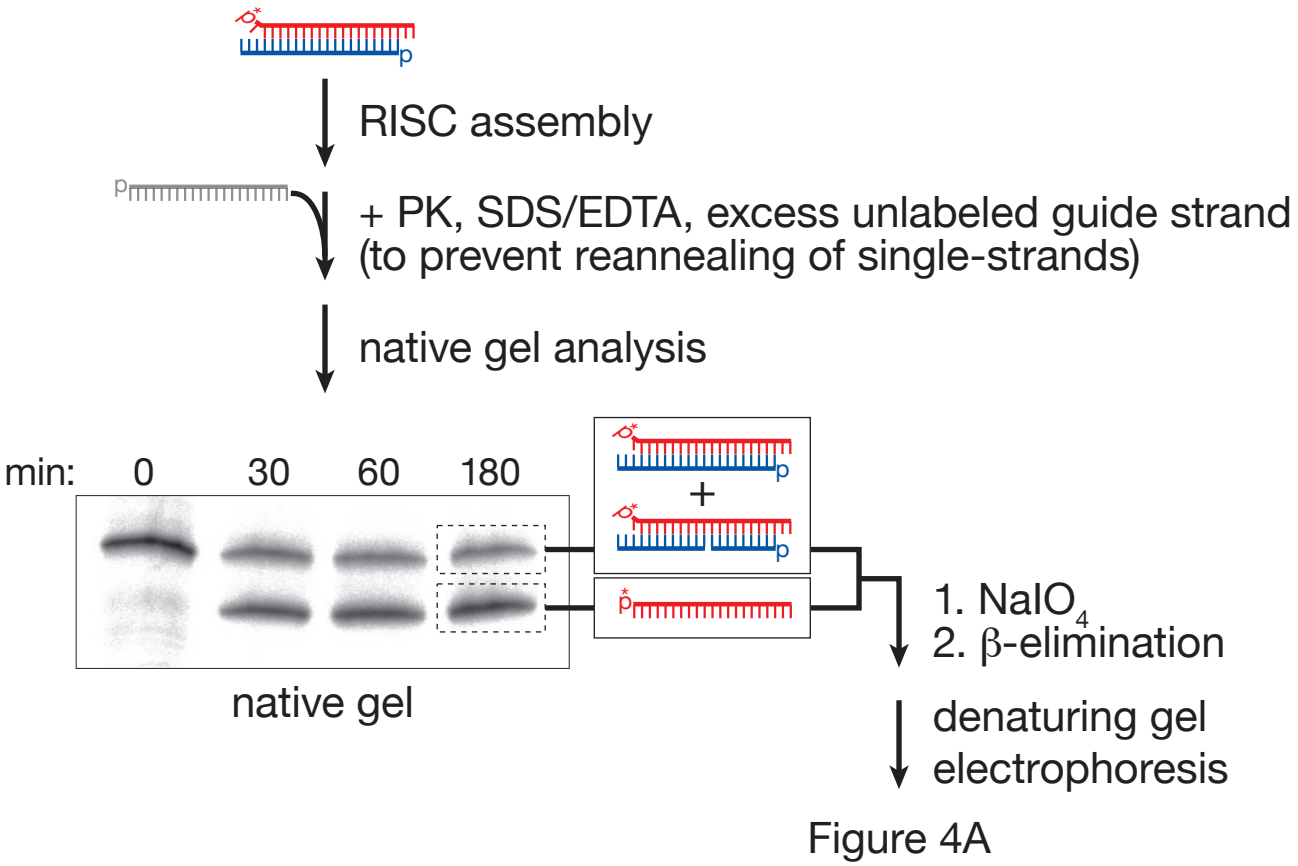

Figure S7. Strategy for testing when during RISC assembly siRNAs are modified at their 3´ termini. Double-stranded siRNA was incubated in embryo lysate to assemble RISC, then the siRNA purified away from protein using a procedure that preserves the single- or double-stranded structure of the siRNA at the time assembly was stopped. The siRNAs were then resolved on a native gel, and the single- and double-stranded siRNA was isolated from the gel, reacted with $NaIO_4$ followed by β-elimination and analyzed by denaturing gel electrophoresis (shown in Figure 4A).

General Discussion

In the preceding chapters, I have discussed in detail a novel mechanism for assembly of Argonaute2-RISC. In the first chapter, I tested the idea that siRNA-unwinding defective wheat germ extract can be rescued with RNAi mutant ovary lysates from *Drosophila*. I found that *armi*, but not *ago2* lysates, could rescue RISC activity from exogenous siRNA suggesting that Ago2 is indeed required for strand separation of the siRNA. In the second chapter, we tested the possibility that Ago2, through its innate endonucleolytic activity, promotes siRNA strand separation. We found that Ago2 cleaves the passenger strand of siRNAs facilitating assembly of mature RISC. Next I tested the ATP requirements for passenger strand cleavage. My findings suggest that ATP is necessary for initiating passenger strand cleavage, as it is required for efficient loading of the siRNA into RLC and pre-RISC (Nykanen et al. 2001; Tomari et al. 2004b; Kim et al. 2007). Moreover, release of the cleaved passenger strand from the guide strand is enhanced by ATP but may not be required. In the fifth chapter, we asked what is the 3´ terminal modification of piRNAs and siRNAs in *Drosophila*. We found that both piRNAs and siRNAs are 2´-*O*-methylated at their 3´ ends by *Drosophila* Hen1 methyltransferase. Further, we found that siRNAs are modified at the final step of RISC assembly, when Ago2-RISC is mature containing only one strand of the original siRNA duplex.

Why is Argonaute2 required for assembly of RISC? Our current model of RISC assembly—facilitated by Ago2 cleavage of the passenger strand—is likely the normal mechanism by which siRNAs are loaded into RISC. When the siRNA duplex contains

artificial modifications or mismatches which block cleavage by Ago2, a bypass mechanism seems to replace the cleavage requirement. This explains how Argonautes that do not retain catalytic function, such as Ago1, 3, and 4 in humans, are eventually loaded into effector complexes. miRNAs were found associated with cleavage-dead Argonaute proteins when ectopically-expressed in human cells (Meister et al. 2004; Liu et al. 2004). Our data suggests that non-cleaving Argonautes could load canonical duplex siRNAs through the bypass mechanism, yet less efficiently than the Ago2-cleavage mechanism. In mice, Ago2 is required for development (Liu et al. 2004)—are other non-cleaving Argonautes necessary? Non-cleaving Argonautes may not be required for direct function (i.e. gene regulation) but may act to sequester some miRNAs to prevent saturation of Ago2. If non-cleaving Argonautes can act on target genes—likely by some mode of translational inhibition—, then these proteins may have lost catalytic residues through evolution in order to dampen both miRNA- and siRNA-guided regulation.

RISC assembly by an Argonaute cleavage-facilitated mechanism is supported by many recent findings. Structural analysis of archeal Argonaute/PIWI proteins showed that the PIWI domain can hold an siRNA:target duplex (Ma et al. 2005; Parker et al. 2005). This data suggests that Ago2 is capable of binding duplex siRNA. However, using recombinant human Ago2, the Hannon and Joshua-Tor groups could only program RISC with single stranded substrate (Liu et al. 2004; Rivas et al. 2005). This suggests that if Ago2 binds duplex siRNA, then it binds with a relatively weak binding constant. There are likely other factors that play a role in programming duplex siRNA into Ago2 such as the RISC-loading complex (RLC) in *Drosophila*. The greater binding capacity of Ago2 for single-stranded siRNA may in fact support our hypothesis. After rapid

cleavage of the passenger strand (<2min), Ago2 itself may pry open the nicked duplex open by binding more tightly to the guide strand than the duplex siRNA. Moreover, recent *in vivo* data from *Schizosaccharomyces* pombe, *Neurospora crassa*, and *Drosophila melanogaster* suggests the catalytic activity of Argonaute is required for assembly of mature RNAi enzyme (Buker et al. 2007; Maiti et al. 2007; Kim et al. 2007).

Conversion of pre-RISC—Ago2 bound to siRNA duplex—to mature single-stranded RISC may require ATP, suggesting that upon ATP hydrolysis, a cofactor removes the cleaved passenger strand from the guide strand (Chapter IV). This cofactor may be an RNA helicase that "unwinds" the remaining siRNA duplex or displaces a portion of Ago2, causing release of the cleavage product. However, the identity of such a cofactor remains elusive. In *Neurospora crassa*, the catalytic activity of the Argonaute homolog QDE-2 is required for generation of single stranded RISC (Maiti et al. 2007). The QDE-2 interacting protein, QIP-1—a putative exonuclease—is also required for RISC assembly, suggesting that an exonuclease acts on the nicked passenger strand after QDE-2 catalysis (Maiti et al. 2007). Does an ATP-dependent exonuclease remove the nicked passenger strand in *Drosophila* and other animals? Interestingly, homologs of *Neurospora* QIP-1 exonuclease exist in all animals including *Drosophila melanogaster*. Genetic and biochemical experiments will need to be done to determine if the QIP-1 homolog or other exonucleases are involved in the conversion of pre-RISC to RISC.

In Chapter V, I discuss data showing that siRNAs—like piRNAs—are 2´-*O*-methylated at their 3´ termini by *Drosophila* HEN1 methyltransferase (DmHen1). DmHen1 methylates only single stranded siRNA that is loaded into Ago2, a late step in the assembly of mature RISC. Our data raises important questions about small

modification by Hen1. First, what is the function of the 3´modification? In plants, HEN1 methylates both miRNAs and siRNAs to prevent polyuridylation of the small RNA, a signal for degradation (Li et al. 2005). Does the 2´-*O*-methyl modification block degradation of siRNAs and piRNAs in animals? This is unlikely the case because steady state levels of endogenous piRNAs are comparable in normal and in *hen1* flies (Chapter V). Perhaps the modification is an anti-determinant for the PAZ domain of Argonautes. Based on structural binding analysis of the PAZ domain of *Drosophila* Ago2, 2´-OH groups—but not 2´-O-methyl—are recognized by the PAZ domain (Lingel et al. 2003; Ma et al. 2004). Inability of Ago2 to bind the 3´end of the guide strand may effect the off-rate of RISC after its guide strand nucleates target RNA (Haley et al. 2004).

Why are piRNAs and siRNAs, but not miRNAs, modified in flies? In the *Drosophila*, the miRNA, piRNAs, and siRNA pathways are separable, meaning that each contain their own distinct RNA substrate and Argonaute protein (Figure I-2 and 3). Does the RNA substrate and/or Argonaute play a role in whether or not the small RNA is modified? Our data suggests that DmHen1 modifies only single stranded substrates, and methylates Ago2-loaded RNA much more efficiently than naked substrate. Does Hen1 interact with Ago2 and piRNA-interacting Argonautes like Piwi and Ago3, but not the miRNA-specific Ago1? Does the 3´ end of miRNAs remain embedded in the PAZ domain of Ago1, preventing methylation by DmHen1?

Is DmHen1 required? Preliminary studies suggest that *hen1* flies have a partial sterility defect; crosses between heterozygous *hen1* males and females produce few progeny compared to wild type, and *hen1* flies also have defects in testes morphology (C. Li and M. Horwich, unpublished observations). Moreover, like Dicer and Argonaute,

Hen1 is highly conserved throughout evolution in organisms that have small gene silencing mechanisms. In fact, miRNAs recently identified from *Chlamydomonas reinhardtii*, miRNAs that are resistant to periodate oxidation, suggesting that a Hen1 homolog exists in green algae (Molnar et al. 2007). In mammals, the mouse homolog of Hen1 was found to methylate piRNAs (Kirino et al. 2007). The remarkable conservation of Hen1 from green algae to mammals by itself illuminates the overall importance of the small RNA methylation activity. Whether the function of 2´-*O*-methylation in plants—blocking degradation pathways (Li et al. 2005)—is conserved in animals will need to be determined.

Gene silencing by small RNAs: an ancient battle between host and virus

We know that RNAi is a natural defense mechanism to fight off viral infection in plants and some animals. Can viruses subvert the RNAi defense mechanism of plants and invertebrates? Viruses have come up with many ways to suppress gene-silencing pathways. First, plant, fungal, and insect viruses produce protein suppressors that block specific steps in RNA silencing pathways (Li and Ding 2006; Ding and Voinnet 2007). *Drosophila* viruses including the flock-house and cricket paralysis viruses encode proteins that block viral dsRNA dicing (Lu et al., 2005; van Rij et al., 2006; Fenner et al., 2007), perhaps by binding A-form RNA helices independent of length (Chao et al. 2005). Structural and biochemical analysis showed that plant viral suppressors block RISC assembly by binding duplex siRNAs, and sequester single-stranded siRNA from AGO1 (Vargason et al., 2003; Ye et al., 2003; Chellappan et al., 2005).

In vertebrates, virus-produced double stranded RNA is generally sensed by innate immune responses such as Toll-like and interferon receptors, and do not elicit an RNAi immunity (Bowie and Fitzgerald 2007). Thus, vertebrate viruses likely do not encode or require protein based silencing suppressors. However, mammalian viruses may have a symbiotic, "love-hate" relationship with the host miRNA pathway (Sarnow et al. 2006)—a pathway thought to evolve from an ancient siRNA pathway. Viruses encode their own miRNAs, which may function by regulating both viral and host gene expression (Pfeffer et al., 2004). Second, virally-encoded RNAs and miRNAs may clog up the miRNA machinery: adenovirus VA1 RNAs inhibit nuclear export and dsRNA processing, as well as produce miRNAs that repress Dicer expression in human cells (Cullen 2006; Stadler et al., in preparation). Moreover, hepatitis C virus seizes the liver-specific miR-122, manipulating the miRNA into supporting viral replication (Jopling et al. 2005).

Conversely, the host has ways to defend itself against viral silencing suppression. RNAi components in *Drosophila* such as Dicer-2, R2D2, and Ago2 have evolved much more rapidly than their miRNA biogenesis counterparts (Obbard et al., 2006), suggesting flies have naturally selected for better immunity. In fact, proteins involved in RNA silencing including Argonautes—viral replicases—and RNA-dependent RNA polymerases—viral polymerases—may have originally been hijacked from viral genomes, ultimately to be used against the virus (Sijen et al., 2001; Liu et al., 2004). In mammals, some endogenous miRNAs regulate genes that are used by the virus; these targets can be both host- and virally-encoded (Lecellier et al., 2005; Otsuka et al., 2007; Triboulet et al. 2007). Moreover, PACT and TRBP—double-stranded binding proteins involved in the PKR immune response—double as assembly factors in mammalian RNA

silencing, further suggesting a role for RNAi in immunity against vertebrate viruses (Gregory et al., 2005; Haase et al., 2005; Lee et al., 2006).

TGS by small RNAs: the preferred mechanism of gene silencing?

In fungi, plants, and animals, small RNAs silence their targets at both the transcriptional and post-transcriptional levels (Broderson and Voinnet 2006; Grewal and Elgin 2007). Is transcriptional silencing the preferred mode of regulation? Is post-transcriptional gene silencing by small RNAs a backup plan for transcriptional gene silencing of the similar loci? If small RNAs can direct DNA and histone methylation at homologous sequences, then is post-transcriptional silencing necessary? In fission yeast, the silencing machinery—RITS—is recruited to nascent transcripts, where is deposits repressive marks on neighboring genomic loci (Buhler et al. 2006). Moreover, is Slicer activity needed for gene regulation? In *S. pombe*, Ago1 slicer-activity is required for its association with chromatin and spreading of H3K9 methylation (Buker et al. 2007). These studies in fission yeast suggest that Ago-cleavage activity may be required only for assembly of mature effector complex, not for downstream target regulation.

Perhaps effector complexes such as RISC or RITS initiate transcriptional silencing through RNA:RNA interaction, likely with nascent transcripts, recruiting chromatin modifying enzymes and other regulatory factors to specific loci. Strategies to tether effector complexes have been somewhat successful in determining the mechanism of gene regulation by small RNAs (Pillai et al. 2004; Buhler et al. 2006). Thus site-specific recruitment of effector complexes may be efficient for gene silencing. Likewise, transcriptional gene silencing would require nuclear compartmentalization of small

RNAs. In plants, miRNAs are processed in the nucleus by Dicer-like proteins and found in both nuclear and cytoplasmic fractions (Papp et al., 2003; Dunoyer et al. 2004). Two plant miRNAs, *miR165* and *miR173*, direct methylation of the template DNA of their predicted targets (Bao et al. 2004; Allen et al. 2005). However, *cis* methylation by *miR165* occurs at sites kilobases away from the original target sites suggesting that the nascent target RNA is cleaved first by the (primary) miRNA (Bao et al. 2004). Cleaved transcript is copied into dsRNA by an RdRP to be further processed into (secondary) 24 nt-siRNAs that direct DNA methylation (Zilberman et al., 2003; Dunoyer et al., 2004). Is cleavage activity from *miR165*- and *miR173*-RISC required for methylation in plants? In *C. elegans*, slicer activity is not necessary for production of secondary siRNAs. Secondary siRNAs (or short transcripts) are produced from primary siRNAs targets that are not cleaved (Sijen et al. 2007), further perpetuating the "recruitment hypothesis" as the primary function of small RNA-guided complexes.

In animals, piRNAs are loaded into Piwi— a *Drosophila* protein that localize to the nucleus (Cox et al., 2000; Brennecke et al. 2007). Interestingly, piRNAs silence transposons (Vagin et al., 2006; Saito et al. 2006; Carmell et al. 2007; Brennecke et al. 2007); however the mode of regulation—post-transcriptional or transcriptional—is not known. Loss of the Piwi proteins, Mili and Miwi2, in mouse testis resulted in demethylation of DNA specific to retrotransposon loci (Aravin et al. 2007; Carmell et al. 2007). Is the piwi-transposon silencing mechanism conserved in other animals? In *C. elegans*, germline small RNAs silence Tc1 transposons—loci from which they are produced—in a manner dependent on RNAi components (Sijen and Plasterk 2003), yet no Argonaute protein was specified to be involved in Tc1 silencing. Are germ-line

specific small RNAs associated with CePRG-1 and PRG-2—piwi-homologs found in *C. elegans* (Tolia and Joshua-Tor, 2007)? Likewise, are the longer, 3´ modified class of small RNAs which were discovered recently (Ruby et al., 2006) responsible for silencing transposons in worms? Immunoprecipitation and *in situ* hybridation experiments should be done to determine if piRNA-like classes exist in *C. elegans*.

If the miRNA pathway originated from an ancient RNAi mechanism, then where did piRNAs come from? piRNAs derive from discrete genomic loci as single-stranded transcripts, presumably by a Dicer-independent mechanism (Figure I-4). This differs from both the miRNA and siRNA which are born as double-stranded precursors (Figure I-2). In flies, the siRNA-producing Dicer-2 is not required for piRNA production (Vagin et al., 2006). However, it is not known whether the miRNA-specific Dicer-1 or the nuclear RNaseIII Drosha plays a role in piRNA production in flies. Moreover, Drosha may be required for production of piRNAs in vertebrates as Dicer is not required for the production of piRNAs in zebrafish (Houwing et al., 2007). The "ping-pong" hypothesis for piRNA biogenesis explains only how these small RNPs are amplified to silence transposable elements (Brennecke et al., 2007; Aravin et al., 2007) but fail to explain how initial complexes are produced.

piRNAs may have emerged rapidly from an the siRNA pathway, hijacking necessary components in response to transposon invasion of the germline. piRNAs are born from single-stranded transcripts: the single-stranded nature of piRNAs may be a mechanism to bypass the immune response against dsRNA in animals. piRNAs production could also require an RNA-dependent RNA polymerase-like activity—even

though flies and vertebrates are thought not to encode such an activity (Schwarz et al. 2002).

Are scRNAs (Figure I-5) early relatives of piRNAs? There are some similarities between these two classes of small RNA: first, they both reside in a piwi-clade Argonaute protein. Twi1, the protein required for scRNA stability and function is a distant relative of Drosophila Aubergine and Piwi, suggesting that scRNAs can use both Dicer-like and "ping-pong" cleavage mechanisms for biogenesis (Mochizuki and Gorovsky 2004; Mochizuki and Gorovsky 2005; Brennecke et al. 2007). Secondly, piRNAs and scRNAs may direct chromatin modification by DNA and histone methylation respectively (Aravin et al. 2007; Liu et al. 2007). Furthermore, both are germline- and nuclear-specific small RNAs, mature forms of which have the same nucleotide length—between 28 to 30 nucleotides (Cox et al. 2000; Mochizuki et al. 2002; Brennecke et al. 2007). Although piRNAs may not use Dicer or DNA elimination as part of their silencing machine, they may have kept a similar mechanism to protect the germ-line from parasitic elements such as transposons.

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APPENDIX:

A Protein Sensor for siRNA asymmetry

Primer: Small Silencing RNAs

Splinted Ligation using Small Phosphorothioate-Modified Oligoribonucleotides

The siRNA-infected cells were assessed for changes in Ca^{2+} oscillation patterns. Average Ca^{2+} signals from Jurkat cells infected with TRPM4 siRNA (n = 64 cells, 5 experiments) and control siRNA (n = 33 cells, 4 experiments) were determined (Fig. 4G). Jurkat cells infected with TRPM4 siRNA exhibited a more prolonged sustained Ca^{2+} influx as compared to cells infected with the siRNA control, which exhibit oscillatory changes typical of wt-Jurkat cells (compare Fig. 4, E and F). PHA stimulation of these cells resulted in a twofold increase in the amount of IL-2 secreted by the TRPM4 siRNA–infected cells as compared with the amount of IL-2 secreted by the control cells (Fig. 4H).

In summary, our results establish that TRPM4 is a previously unrecognized ion channel in Jurkat T cells with a profound influence on Ca^{2+} signaling. Molecular suppression of TRPM4 converts oscillatory changes of $[Ca^{2+}]_i$ into long-lasting sustained elevations in Ca^{2+} and leads to augmented IL-2 production. It is conceivable that this effect occurs physiologically in cells that express the short splice variant TRPM4a, which is of a similar length to ΔN-TRPM4 and could therefore act as a native dominant negative subunit. In electrically nonexcitable cells, TRPM4 would tend to reduce Ca^{2+} influx by depolarizing the membrane potential and reducing the driving force for Ca^{2+} entry through store-operated CRAC (Ca^{2+} release–activated Ca^{2+}) channels.

The molecular and electrophysiological identification of TRPM4 in Jurkat T cells may call for a reinterpretation of the interplay of ionic currents that shape intracellular Ca^{2+} signals (*4*, *16*, *19*). We propose that TRPM4 acts in concert with CRAC, Kv1.3, and K_{Ca} channels to control $[Ca^{2+}]_i$ oscillations in lymphocytes through oscillatory changes in membrane potential according to the following model.

At rest, the lymphocyte membrane potential is around –60 mV, owing to the basal activity of K^+ channels (*20*). Engagement of T cell receptors induces phospholipase C–mediated production of $InsP_3$ (inositol 1,4, 5-trisphosphate), which causes Ca^{2+} release and activation of store-operated CRAC channels. Current models of Ca^{2+} oscillations in lymphocytes (*19*) propose that the I_{CRAC}-mediated Ca^{2+} influx triggers the activation of Ca^{2+}-activated K^+ channels, which provides the driving force for Ca^{2+} entry by hyperpolarizing the membrane potential until $[Ca^{2+}]_i$ reaches a high-enough level to inhibit I_{CRAC}. As Ca^{2+} entry through I_{CRAC} is reduced, $[Ca^{2+}]_i$ falls until it reaches a level that removes the negative feedback on I_{CRAC}, and the cycle resumes by increasing Ca^{2+} entry through I_{CRAC}. This model lacks a strong depolarizing conductance that would be required to recruit voltage-dependent Kv1.3 channels present in T cells and could account for the observed oscillations in membrane potential (*20*, *21*). The $[Ca^{2+}]_i$-dependent activation of TRPM4 channels may provide this mechanism by becoming activated at around the peak of an oscillatory Ca^{2+} transient, causing the membrane potential to depolarize and thereby substantially reducing the driving force for Ca^{2+} influx. The depolarization would then recruit voltage-dependent K^+ currents (Kv1.3), which would tend to repolarize the membrane potential and also aid in the closure of TRPM4 channels, because the open probability of TRPM4 channels is reduced at negative membrane voltages (*5*, *6*, *8*). The repolarization would reestablish the driving force for Ca^{2+} influx through I_{CRAC} so that the next oscillation in $[Ca^{2+}]_i$ can take place.

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Materials and Methods

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A Protein Sensor for siRNA Asymmetry

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To act as guides in the RNA interference (RNAi) pathway, small interfering RNAs (siRNAs) must be unwound into their component strands, then assembled with proteins to form the RNA-induced silencing complex (RISC), which catalyzes target messenger RNA cleavage. Thermodynamic differences in the base-pairing stabilities of the 5′ ends of the two ~21-nucleotide siRNA strands determine which siRNA strand is assembled into the RISC. We show that in *Drosophila*, the orientation of the Dicer-2/R2D2 protein heterodimer on the siRNA duplex determines which siRNA strand associates with the core RISC protein Argonaute 2. R2D2 binds the siRNA end with the greatest double-stranded character, thereby orienting the heterodimer on the siRNA duplex. Strong R2D2 binding requires a 5′-phosphate on the siRNA strand that is excluded from the RISC. Thus, R2D2 is both a protein sensor for siRNA thermodynamic asymmetry and a licensing factor for entry of authentic siRNAs into the RNAi pathway.

In *Drosophila* lysates, siRNAs are loaded into the RISC by an ordered pathway in which one of the two siRNA strands, the guide strand, is assembled into the RISC, whereas the other strand, the passenger strand, is excluded and destroyed (*1*–*14*). A central step in RISC assembly is formation of the RISC-loading complex [RLC, previously designated complex A (*13*)], which contains double-stranded siRNA, the double-stranded RNA binding protein R2D2, and Dicer-2 (Dcr-2), as well as additional unidentified proteins. The function of Dicer in loading siRNA into the RISC is distinct from its role in generating siRNA from long double-stranded RNA (dsRNA) (*10*, *15*). Both R2D2 and Dcr-2 are

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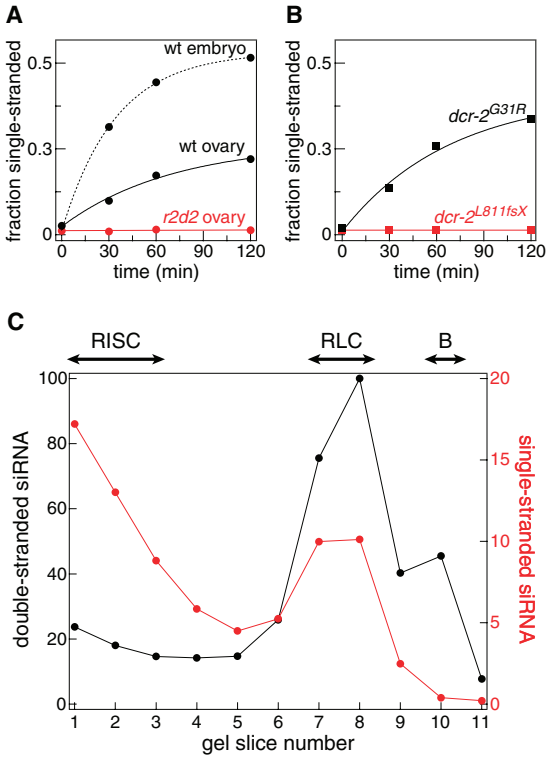

Fig. 1. The RISC-loading complex (RLC) initiates siRNA unwinding. (**A**) *r2d2* mutant ovary lysates, which cannot assemble RLC, do not unwind siRNA (wt, wild type). (**B**) siRNA unwinding was defective in ovary lysate from a mutation that disrupts all known Dcr-2 functions (L811fsX), including RLC assembly (*10, 12, 18*), but was normal for a point mutation in *dcr-2* ($Gly^{31} \rightarrow Arg$, G31R) that disrupts its function in dicing long dsRNA into siRNA, but not RISC assembly (*10, 12*). (**C**) The RLC, which is composed largely of double-stranded siRNA, also contains single-stranded siRNA. Note the different scales for the relative amounts of single- and double-stranded siRNA.

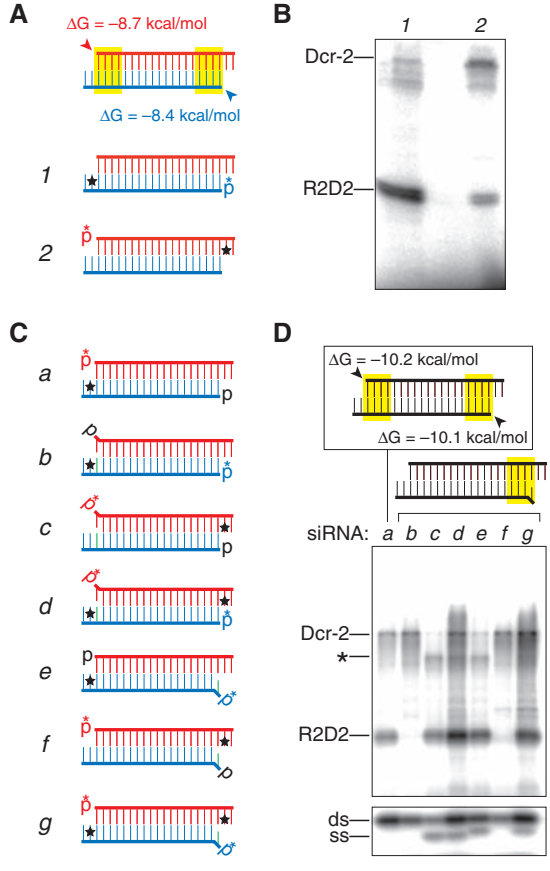

Fig. 2. Asymmetric binding of the Dcr-2/R2D2 heterodimer to siRNA duplexes in *Drosophila* embryo lysate. (**A**) Structure of the fully base-paired but asymmetric luciferase siRNA duplex used in (B) for protein-siRNA photocrosslinking. The local thermodynamic stability of the yellow highlighted base pairs is indicated here and in (D). Stars denote 5-iodouracil; an asterisk denotes a 5′-[^{32}P]phosphate. (**B**) The blue strand generates about 5 times as much RISC as the red; more Dcr-2 photocrosslinks to the 5-iodouracil nearest the less thermodynamically stable end, whereas more R2D2 photocrosslinks to the 5-iodouracil nearest the more thermodynamically stable end. (**C**) The series of 5-iodouracil substituted siRNAs used in (D). siRNA *a* is thermodynamically symmetric. siRNAs *b* to *g* contain a single unpaired nucleotide at the 5′ end of one strand, making them highly asymmetric (*7*). For siRNAs *b* to *d*, the red strand serves as the guide strand, whereas the blue strand serves as the guide for siRNAs *e* to *g*. (**D**) For the functionally asymmetric siRNAs, Dcr-2 binding was detected nearest the 5′ end of the guide strand, whereas R2D2 was detected near the 5′ end of the passenger strand, the more thermodynamically stable end. The lower panel shows the ^{32}P-radiolabeled siRNA strands at the bottom of the same gel. Single-stranded siRNA was detected only when the labeled strand served as a guide strand and entered the RISC. The asterisk indicates a photocrosslink to Ago2.

required to form RLC (*13*) and to unwind siRNA (Fig. 1, A and B), but recombinant Dcr-2/R2D2 heterodimer or Dcr-2 alone cannot catalyze siRNA unwinding (fig. S1). Thus, the Dcr-2/R2D2 heterodimer is necessary but not sufficient to unwind siRNA.

If siRNA unwinding is initiated in the RLC, then the RLC should contain some single-stranded siRNA. To test this idea, we briefly incubated siRNA duplex in lysate, resolved the complexes formed by native gel electrophoresis, divided the gel into 11 parts, and analyzed the structure of the siRNA in each gel slice (fig. S2A). Consistent with our previous findings, a peak of double-stranded siRNA comigrated with both the RLC and complex B, which is thought to be a precursor to RLC (*13*) (Fig. 1C). A small peak of single-stranded siRNA also comigrated with the RLC, but not with complex B (Fig. 1C), which suggests that the RLC initiates siRNA unwinding. Similar peaks of single-stranded siRNA comigrated with the RLC for the passenger strand of this siRNA and for the guide and passenger strands of a second siRNA (fig. S2B). We conclude that the RLC initiates siRNA unwinding.

The RLC also senses siRNA thermodynamic asymmetry, thereby determining which strand enters the RISC. siRNA containing 5-iodouracil at the 20th nucleotide (p20) can be photocrosslinked to R2D2 and Dcr-2 (*13*). Photocrosslinking is position-specific: An siRNA containing 5-iodouracil at position 12 was not cross-linked to R2D2 or Dcr-2 (*13*). Photocrosslinking attaches the radiolabel of the siRNA to the protein, identifying proteins that lie near p20 of the substituted siRNA strand. We evaluated the relative efficiency of photocrosslinking to R2D2 and Dcr-2 for three types of siRNA (Fig. 2) (table S1): a luciferase-specific siRNA whose sequence makes the 5′ end of the antisense strand less thermodynamically stable than the 5′ end of the sense strand; a nearly symmetric siRNA targeting human *Zn*, *Cu superoxide dismutase 1* (*sod1*), in which the stabilities of the 5′ ends are essentially the same; and a series of highly asymmetric *sod1*-directed siRNAs in which the first nucleotide of the guide strand is mismatched to the passenger strand, causing the guide strand to be loaded into the RISC almost exclusively. When we used the partially asymmetric luciferase-specific siRNA, R2D2 was more efficiently photocrosslinked when the 5-iodouracil was on the strand more frequently incorporated into the RISC, whereas when the 5-iodouracil was on the strand less often incorporated into the RISC, Dcr-2 was more efficiently photocrosslinked (Fig. 2, A and B). Because a 5-iodouracil at p20 of one siRNA strand is near the 5′ end of the other strand, Dcr-2 must lie near the 5′ end of the strand entering the RISC (the guide strand), whereas

R2D2 binds near the 5′ end of the strand destined for destruction.

When we used the symmetric *sod1* siRNA (Fig. 2, C and D, siRNA *a*), Dcr-2 and R2D2 were photocrosslinked with nearly equal efficiency to the 5-iodouracil strand (Fig. 2D, siRNA *a*); this finding suggests that each protein binds about half the time to one or the other end of the siRNA. In contrast, when we used derivatives of this siRNA that contained single-nucleotide mismatches that made them highly asymmetric, the 5-iodouracil strand was photocrosslinked to either Dcr-2 or R2D2, but not to both (Fig. 2, C and D, siRNAs *b* and *c*). With the asymmetric siRNA sequence, the photocrosslinking data suggest that Dcr-2 is almost always near the 5′ end of the guide strand and R2D2 near the 5′ end of the passenger strand. As expected when both siRNA strands contained p20 5-iodouracil and 5′-[^{32}P]phosphate groups, both proteins were photocrosslinked (Fig. 2, C and D, siRNA *d*). When we used a reciprocal series of siRNAs in which the strands assembled into and excluded from the RISC were reversed (Fig. 2, C and D, siRNAs *e*, *f*, and *g*), Dcr-2 was again found near the 5′ end of the guide strand and R2D2 near the 5′ end of the passenger strand.

Purified, recombinant Dcr-2/R2D2 heterodimer alone can also sense the thermodynamic stabilities of the ends of an siRNA duplex. At physiologically relevant concentrations of the proteins (*16*), photocrosslinking reflected siRNA asymmetry (Fig. 3A). Like heterodimer binding to an siRNA (*17*), differential photocrosslinking of recombinant Dcr-2/R2D2 heterodimer to an siRNA (*18*) did not require adenosine triphosphate (ATP). In contrast, formation of the RLC requires ATP (*13*). The orientation of Dcr-2 and R2D2 on the siRNA duplex was less asymmetric for the recombinant heterodimer than for embryo lysate (compare Figs. 2D and 3A). We propose that siRNA asymmetry is initially sensed by the Dcr-2/R2D2 heterodimer in an ATP-independent manner but is later amplified by the ATP-dependent action of other proteins.

Photocrosslinking of R2D2, but not Dcr-2, to the two ends of an siRNA duplex was influenced by the presence of a 5′-phosphate group on the siRNA. We prepared a series of highly asymmetric siRNAs in which the strand containing the p20 5-iodouracil was radiolabeled with ^{32}P at the 5′ end and the other strand contained either a 5′-hydroxyl or 5′-phosphate group (Fig. 3B). In four trials, R2D2 photocrosslinking to the nearby p20 5-iodouracil of the guide strand was greater by a factor of 4.6 ± 0.4 (average ± SD) when the passenger strand contained a 5′-phosphate rather than a hydroxyl group (Fig. 3C, left, siRNAs *c* and *e*). R2D2 photocrosslinking in ATP-depleted embryo lysate likewise required a 5′-phosphate at the more thermodynamically stable siRNA end (Fig. 3C, right, siRNAs *c* and *e*). Thus, R2D2 can sense two aspects of siRNA structure: the stability of an siRNA 5′ end, and the presence of a 5′-phosphate group. In contrast, Dcr-2 photocrosslinking was unperturbed by a 5′-hydroxyl group on the guide strand, both for the purified protein and in ATP-depleted lysate (Fig. 3C, siRNAs *b* and *f*).

Active siRNAs contain 5′-phosphate groups on both strands (*3*, *11*, *19*–*21*). A 5′-phosphate on the guide strand is essential for siRNA function, but blocking 5′-phosphorylation of the passenger strand impairs rather than eliminates siRNA activity (*11*). Our results suggest a molecular explanation for this observation: A 5′-phosphate on the passenger strand enhances R2D2 binding, thereby facilitating efficient incorporation of an siRNA into the RLC and consequently into the RISC. Thus, R2D2 is a licensing factor that ensures that only authentic siRNAs enter the RNAi pathway in *Drosophila*.

Dcr-2 alone does not efficiently bind siRNA (*17*), nor can Dcr-2 alone be photocrosslinked to any of the siRNAs in this study (*18*). Taken together, these results and the data presented here suggest that orientation of the Dcr-2/R2D2 heterodimer is determined largely by R2D2 binding to the siRNA end with the most double-stranded character. This binding is presumably mediated by one or both of the R2D2 double-stranded RNA binding domains. A 5′ mismatch on an siRNA strand may therefore be an antideterminant for R2D2 binding, acting to direct the R2D2 protein to the 5′ end of the passenger

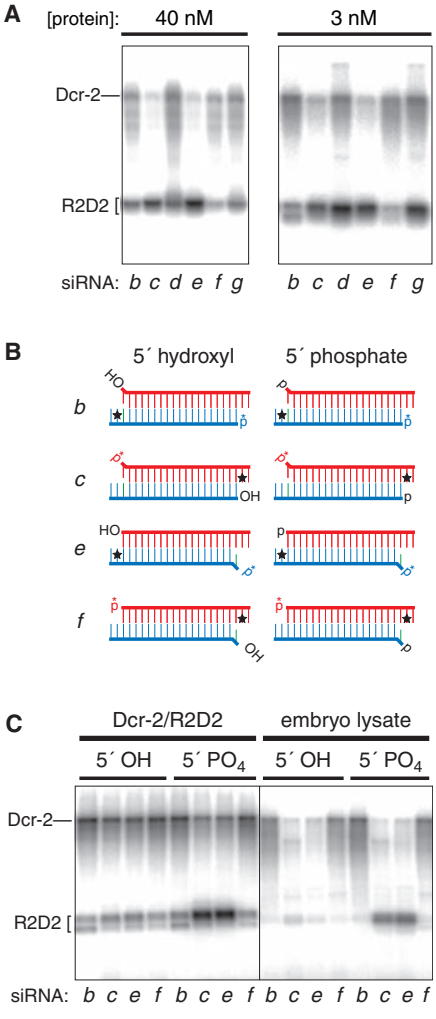

Fig. 3. The Dcr-2/R2D2 heterodimer alone can sense the asymmetry of an siRNA. (**A**) Photocrosslinking of recombinant Dcr-2/R2D2 heterodimer to the series of asymmetric siRNAs in Fig. 2C. (**B**) Structure of the siRNAs used in (C). The siRNAs all contained a single 5′-[^{32}P]phosphate on one strand and either a 5′-hydroxyl or 5′-phosphate group on the other. The siRNA sequences were as in Fig. 2C. (**C**) R2D2 senses the presence of a 5′-phosphate on the passenger strand. R2D2 photocrosslinking to the 5-iodouracil nearest the 5′ end of the passenger strand was reduced when the 5′ end of the passenger strand contained a 5′-hydroxyl rather than a 5′-phosphate group (siRNAs *c* and *e*); photocrosslinking of Dcr-2 was unaltered by the presence or absence of a 5′-phosphate on the guide strand (siRNAs *b* and *f*).

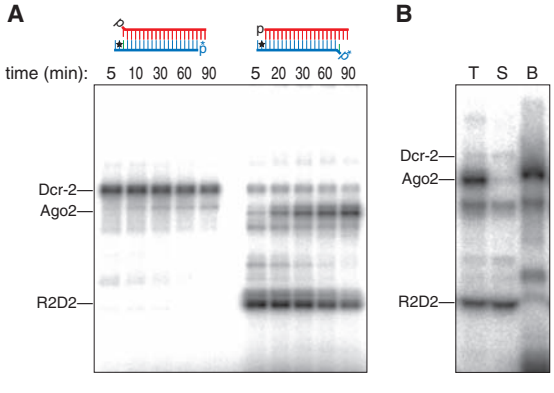

Fig. 4. Exchange of R2D2 for Ago2 at the 3′ end of the siRNA guide strand. (**A**) ^{32}P-radiolabeled siRNAs *b* and *e* (Fig. 2B) were incubated with embryo lysate in the presence of ATP for the times indicated, and binding of proteins near the 3′ end of the siRNA guide and passenger strands was monitored by photocrosslinking. Indicated times include the 4 min during which the sample was exposed to ultraviolet irradiation at room temperature. (**B**) ^{32}P-radiolabeled siRNA *c* was incubated with embryo lysate and photocrosslinked. The photocrosslinked proteins were then captured with a 2′-*O*-methyl oligonucleotide complementary to the siRNA guide strand. T, total reaction before incubation with the tethered oligonucleotide; S, supernatant after incubation; B, siRNA-photocrosslinked proteins bound to the tethered oligonucleotide.

strand and positioning Dcr-2 near the 5′ end of the strand to be loaded into the RISC. In this model, R2D2, as a component of the Dcr-2/R2D2 heterodimer, is the primary protein sensor of siRNA thermodynamic asymmetry.

How does the RLC, with the Dcr-2/R2D2 heterodimer positioned asymmetrically on the siRNA, progress to the RISC? Argonaute 2 (Ago2) is a ~130-kD protein that is a core component of the RISC (*22*) and is required for siRNA unwinding (*14*). We found that a ~130-kD protein was crosslinked to siRNA when the guide strand contained 5-iodouracil at p20 (asterisk in Fig. 2C, siRNAs *c*, *d*, *e*, and *g*). The ~130-kD protein was photocrosslinked only to the guide strand of the siRNA (Fig. 4), which suggests that this protein is a component of the RISC. The ~130-kD protein was immunoprecipitated with antibodies to Ago2 but not to Ago1 (fig. S3A) and was not observed in embryos lacking both maternal and zygotic Ago2 (*ago2*^{414}, fig. S3B). Thus, the ~130-kD protein is Ago2. When R2D2 and Ago2 were photocrosslinked to siRNAs *b* or *e* (which contain 5-iodouracil at p20 of the passenger or the guide strand), R2D2 was bound to the 3′ end of the guide strand and Dcr-2 to the 3′ end of the passenger strand at early times in the reaction (Fig. 4A). Later, binding of R2D2 and Dcr-2 decreased concurrently, accompanied by a corresponding increase in binding of Ago2 to the 3′ end of the guide strand. In *ago2*^{414} lysates, R2D2 binding to the 3′ end of the guide strand and Dcr-2 binding to the 3′ end of the passenger strand did not decrease with time (fig. S4A); this finding suggests that binding of Ago2 facilitates the release of the heterodimer from siRNA.

The siRNA bound by Ago2 is single-stranded, because Ago2, when photocrosslinked to siRNA, was captured by a tethered 2′-*O*-methyl oligonucleotide complementary to the siRNA guide strand (Fig. 4B) (*23*), as has been observed for the RISC (*7*, *23*–*25*). R2D2 was not captured by the 2′-*O*-methyl oligonucleotide, but was instead recovered in the supernatant, consistent with R2D2 binding of double-stranded siRNA.

Our data suggest a model for RISC assembly. First, R2D2 orients the Dcr-2/R2D2 heterodimer on the siRNA within the RLC. As siRNA unwinding proceeds, the heterodimer is exchanged for Ago2, the core component of the RISC. Indeed, we cannot detect single-stranded siRNA in the RLC assembled in *ago2*^{414} lysate (fig. S4, B and C). We hypothesize that unwinding occurs only when Ago2 is available, so that siRNA in the RLC is unwound only when the RISC can be assembled.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/306/5700/1377/DC1
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The Human Polyomavirus, JCV, Uses Serotonin Receptors to Infect Cells

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The human polyomavirus, JCV, causes the fatal demyelinating disease progressive multifocal leukoencephalopathy in immunocompromised patients. We found that the serotonergic receptor $5HT_{2A}$R could act as the cellular receptor for JCV on human glial cells. The $5HT_{2A}$ receptor antagonists inhibited JCV infection, and monoclonal antibodies directed at $5HT_{2A}$ receptors blocked infection of glial cells by JCV, but not by SV40. Transfection of $5HT_{2A}$ receptor–negative HeLa cells with a $5HT_{2A}$ receptor rescued virus infection, and this infection was blocked by antibody to the $5HT_{2A}$ receptor. A tagged $5HT_{2A}$ receptor colocalized with labeled JCV in an endosomal compartment following internalization. Serotonin receptor antagonists may thus be useful in the treatment of progressive multifocal leukoencephalopathy.

The incidence of progressive multifocal leukoencephalopathy (PML) has increased 50-fold since 1979 and now affects nearly 1 in every 200,000 persons (*1*). The disease is due to infection of oligodendrocytes by the common human polyomavirus, JCV (*2*). Initial infection with JCV occurs early in childhood and eventually reaches a seroprevalence of between 70 and 80% in the adult population. The initial infection is subclinical, and the virus establishes a lifelong persistent infection. At any given time, ~5% of the population is actively excreting virus in the urine, and JCV is a frequent contaminant of untreated human sewage (*3*). PML occurs almost exclusively in severely immunosuppressed patients. The majority of cases occur in patients with AIDS, and to date there is no effective treatment (*4*). PML is initiated when JCV traffics from peripheral sites, such as the kidney and lymphoid organs, to the central nervous system (CNS) by unknown mechanisms. There is a strong association between JCV and human B lymphocytes, and the virus may traffic to the CNS in an

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Supporting Online Material

Materials and Methods

General Methods

Native gel analysis of RISC assembly and photocrosslinking of 5-iodo-uracil-containing siRNAs were performed as described (*1*). In photocrosslinking experiments, the siRNA concentration was 25 nM unless otherwise noted, and the photocrosslinked proteins were resolved by 4-20% gradient SDS-polyacrylamide gel electrophoresis (Criterion precast gel system; BioRad). For the photocrosslinking experiments, the incubation time and the UV irradiation time were as follows: Fig. 2 and Fig. 3A, incubation for 1 h and photocrosslinking for 15 min; Fig. 3A, incubation for 10 min and photocrosslinking for 10 min; Fig. 3C, incubation for 1 min and photocrosslinking for 4 min; Fig. 4A and Fig. S4A, incubation as indicated including photocrosslinking for 4 min; Fig. 4B and Fig. S3B, incubation for 86 min and photocrosslinking for 4 min. *Drosophila* stocks were as reported elsewhere (*2-4*).

Unwinding assay with increasing concentration of trap RNA

siRNA unwinding was as described previously (5) except that the guide strand of the *Pp* luciferase-specific siRNA (see siRNA 1 in Supplementary Table 1) was 5′ radiolabeled using T4 polynucleotide kinase, annealed to a two-fold excess of passenger strand and double-stranded siRNA purified by native polyacrylamide gel electrophoresis. 50 nM purified, duplex siRNA was incubated in a standard RNAi reaction with lysate or recombinant proteins (15 nM) at 25°C in the presence of unlabeled guide strand as indicated. Reactions were stopped by the addition of Proteinase K Buffer (100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, and 1% (w/v) SDS) containing 1 mg/ml

Proteinase K and 20 µg glycogen, incubated 30 min at 25°C, and the RNA precipitated with three volumes of absolute ethanol at –20°C. The precipitate was collected by centrifugation, washed once with 80% (v/v) ethanol, redissolved in native loading dye (3% w/v Ficoll, 0.04% w/v Bromophenol Blue, 2 mM Tris-HCl (pH 7.4)), and analyzed by electrophoresis at 5 W at 4°C through a native 15% polyacrylamide gel (29:1, acrylamide:bisacrylamide).

Detection of single-stranded siRNA in RLC

^{32}P-radiolabeled siRNA was incubated with lysate, separated by native gel electrophoresis (*1*), and then fractionated by cutting the gel at 6 mm intervals, excluding the well. The gel slices were soaked in Proteinase K buffer containing 1 mg/ml Proteinase K, 20 µg glycogen, and 100 nM unlabeled RNA corresponding to the sequence of the radiolabeled strand. The eluted RNA was then precipitated with ice-cold ethanol. The RNA was then redissolved in native loading dye and analyzed by electrophoresis through a native 20% polyacrylamide gel.

Purification of recombinant Dcr-2 and Dcr-2/R2D2

Sf21 insect cells were infected with His_6-Dcr-2 virus alone or together with His_6-R2D2 virus (*4*). Cell pellets from one liter culture were lysed in HM buffer (10 mM HEPES, 2 mM $Mg(OAc)_2$, 5% Glycerol and 5 mM DTT); centrifuged at 100,000 x g for 1 h at 4°C; and the supernatant purified by HisTrap (Amersham) chromatography in HM buffer, eluting with a 0-400 mM imidazole gradient. The peak of recombinant protein was further purified by HiTrap Q (Amersham) chromatography and HiTrap S (Amersham) chromatography. Each column was eluted with a 10-350 mM KCl gradient. The concentration of recombinant protein was determined by quantitative amino acid analysis

of protein in a slice from an SDS-polyacrylamide gel (Keck Biotechnology Resource Laboratory). Both the stoichiometry of the purified Dcr-2/R2D2 heterodimer (1:1.01) and its elution profile in gel filtration suggest that it was almost exclusively heterodimeric. For photocrosslinking experiments, purified recombinant enzymes were diluted in lysis buffer containing 5 mg/ml BSA (New England Biolabs).

Determining R2D2 concentration in vivo and in lysate

Drosophila 0-2 h embryos were individually hand collected, dechorionated with 50% (v/v) bleach, and lysed in 1X SDS-PAGE loading buffer. A dilution series of recombinant Dcr-2/R2D2, standard embryo lysate and hand-collected total embryos were loaded on the same SDS-PAGE gel. Quantitative Western analysis with anti-R2D2 antibody (*4*) was performed as described (*6*) using recombinant R2D2 concentration standards. The R2D2 bands were quantified using a LAS-3000 (Fuji), and the intraembryonic concentration of R2D2 (~46 nM in vivo in syncitial blastoderm embryos and ~8 nM in lysate) was calculated assuming that the intracellular volume of an embryo is equal to the average volume of water in a single embryo, 7.3 nl (*7*).

Immunoprecipitation

siRNA with 5-iodo-uracil at position 1 on the guide strand was used for crosslinking. Immunoprecipitation by anti-Ago1 (*8*) and anti-Ago2 antisera (*9*) was performed as described before (*1*), except that Protein A paramagnetic beads (Dynabead Protein A; Dynal Biotech) were used instead of Protein A agarose beads.

Capture of crosslinked proteins by tethered 2′-O-Methyl oligonucleotide

^{32}P-radiolabeled siRNA *c* was incubated with embryo lysate for 86 min, then crosslinked by irradiation for 4 min at room temperature. The reaction was then incubated with a 31 nt 2′-*O*-methyl oligonucleotide that contained a sequence complementary to the siRNA guide strand (5′-biotin-AUGUUGGAGACUUGGGCAAUGUGACUGCUGA-3′), as described (*10*). The 2′-*O*-methyl oligonucleotide was tethered to paramagnetic streptavidin beads via a 5′ biotin group.

Supporting Online Table S1

siRNA *1*	CGUACGCGGAAUACUUCGAUU ||||||||||||||||||| GUGCAUGCGCCUUAUGAAGCU-p*
siRNA *2*	p*-CGUACGCGGAAUACUUCGAUU ||||||||||||||||||| GUGCAUGCGCCUUAUGAAGCU
siRNA *a*	GUCACAUUGCCCAAGUCUCTT ||||||||||||||||||| TUCAGUGUAACGGGUUCAGAG-p*
siRNA *b*	UUCACAUUGCCCAAGUCUCTT ||||||||||||||||||| TUCAGUGUAACGGGUUCAGAG-p*
siRNA *c*	p*-UUCACAUUGCCCAAGUCUCUT ||||||||||||||||||| TTCAGUGUAACGGGUUCAGAG
siRNA *d*	p*-UUCACAUUGCCCAAGUCUCUT ||||||||||||||||||| TUCAGUGUAACGGGUUCAGAG-p*
siRNA *e*	GUCACAUUGCCCAAGUCUATT ||||||||||||||||||| TUCAGUGUAACGGGUUCAGAG-p*
siRNA *f*	p*-GUCACAUUGCCCAAGUCUAUT ||||||||||||||||||| TTCAGUGUAACGGGUUCAGAG
siRNA *g*	p*-GUCACAUUGCCCAAGUCUAUT ||||||||||||||||||| TUCAGUGUAACGGGUUCAGAG-p*

Legend to Supporting Online Table S1.

Table S1. siRNA sequences used in this study. siRNAs *1* and *2* target firefly luciferase, and siRNAs *a-g* target human *sod1*. U indicates 5-iodo-uracil; the 5′-^{32}P radiolabel is marked with an asterisk and highlighted in yellow.

Legends to Supporting Online Figures

Figure S1. Recombinant Dcr-2 and R2D2 proteins alone cannot unwind siRNA duplexes. (A) Schematic of the assay for measuring unwinding of an siRNA in which the guide strand (red) is 5′-^{32}P-radiolabeled. (B) Analysis of siRNA unwinding. We incubated the recombinant proteins with siRNA, ATP, and increasing concentrations of unlabeled RNA having the same sequence as the radiolabeled strand. The guide strand of the siRNA was 5′ ^{32}P-radiolabeled. The unlabeled RNA served to trap any single-stranded siRNA generated by unwinding. After incubation, the proteins were digested with Proteinase K under conditions that preserve siRNA structure, then the siRNA was analyzed on a non-denaturing gel to resolve single-stranded from double-stranded siRNA. In *Drosophila* embryo lysate, siRNAs are unwound and the passenger strand is destroyed, so a 'trap' RNA is not required to detect unwinding. When the siRNA duplex by itself was heat denatured, the presence of increasing amounts of the trap RNA permitted detection of the dissociation siRNA duplex into its component strands. In contrast, no unwinding was detected when physiologically relevant concentrations of Dcr-2 or Dcr-2/R2D2 heterodimer were incubated with the siRNA duplex. The recombinant Dcr-2/R2D2 heterodimer also could not unwind siRNA when the non-radioactive siRNA strand was phosphorylated (*11*). Both protein preparations were highly active for the processing of long dsRNA into siRNA (*11*).

Figure S2. (A) Experimental scheme for detecting siRNA unwinding in the RLC. (B) Four independent trials showing that RLC contains a small amount of single-stranded siRNA. Either the guide or the passenger stand was ^{32}P-radiolabeled for a luciferase siRNA (identical to siRNA *1*, but with uracil in place of 5-iodo-uracil), which is slow to assemble RISC, allowing Complex B to be detected, or for a sod1 siRNA (identical to

siRNA *b*, but with dT in place of 5-iodo-uracil), which assembles RISC more rapidly. The *x*-axis shows the gel slice number, the *y*-axis gives the relative amount of double-stranded or single-stranded siRNA in each band, normalized to the peak of the double-stranded siRNA in RLC. The right upper graph (luciferase siRNA with guide strand labeled) is presented also in Fig. 1C. The sample in the sixth gel slice for luciferase siRNA with a radiolabeled passenger strand was lost.

Figure S3. The ~130 kDa protein photocrosslinked to the ^{32}P-radiolabeled guide strand is Ago2. (A) Immunoprecipitation of proteins photocrosslinked to the siRNA containing a 5-iodo-uracil at position 1 (p1) of the guide strand. p1 siRNA can be photocrosslinked to two different proteins (~110 kDa and ~130 kDa). The ~130 kDa photocrosslinked protein detected with this siRNA comigrated with that observed with the 5-iodo-uracil at p20 (siRNA *c*)(*11*). The ~110 kDa band was immunoprecipitated with anti-Ago1 antisera (*8*), and ~130 kDa band with anti-Ago2 antisera (*9*). (B) ~130 kDa protein photocrosslinked to siRNA *e* (which contains a 5-iodo-uracil at p20 of the guide strand) was not detected in lysates lacking both maternal and zygotic Ago2 (*ago2*^{414}). Both wild-type and *ago2*^{414} (*3*) lysates were prepared from overnight collections of embryos.

Figure S4. (A) Lysates from wild-type (*11*) and *ago2*^{414} overnight embryo collections were incubated with siRNA *b* and *e* in the presence of ATP, and the binding of proteins near the 3′ end of the siRNA passenger and guide strands monitored by photocrosslinking. Indicated times include the four minutes during which the sample was UV irradiated at room temperature. In wild-type lysates, binding of Dcr-2 and R2D2 decreased with time, accompanied by a corresponding increase in binding of Ago2 (*11*). In *ago2*^{414} lysates, Dcr-2 and R2D2 binding did not decrease with time. (B) RISC assembly assay using guide-

strand 5′-^{32}P-radiolabeled luciferase siRNA (identical to siRNA *1*, but with uracil in place of 5-iodo-uracil). (C) The peaks of RISC, RLC and complex B were excised from the agarose gel in (B) and analyzed to determine the structure of the siRNA in the gel slice. Double-stranded and single-strand siRNA from this analysis were separated on a native polyacrylamide gel. No single-strand siRNA was detected in the RLC formed in *ago2*^{414} embryo lysates.

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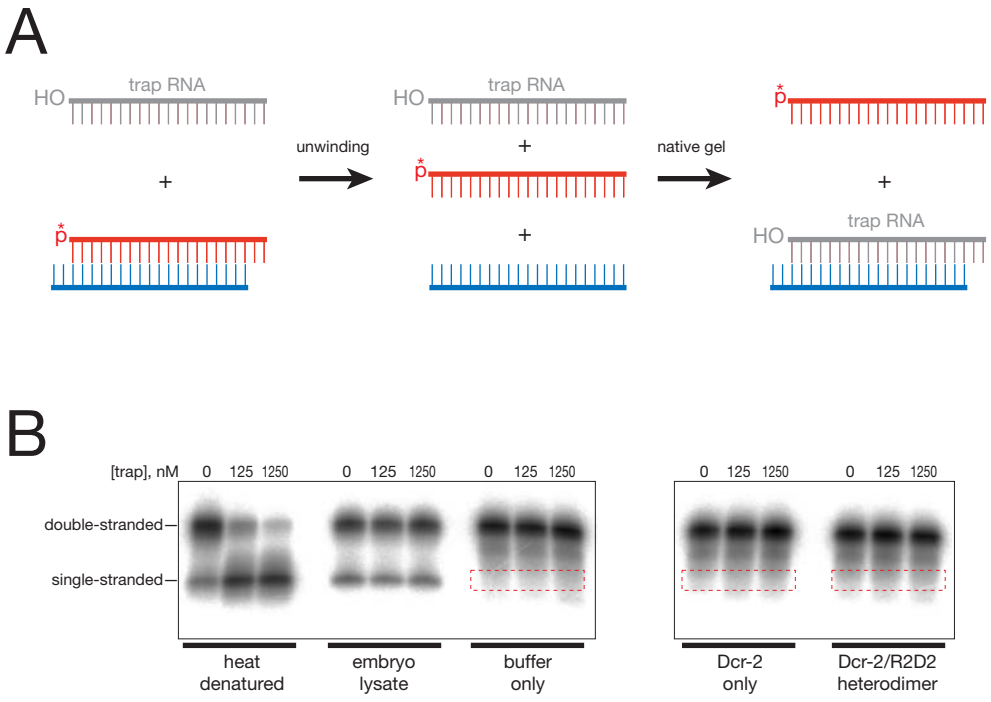
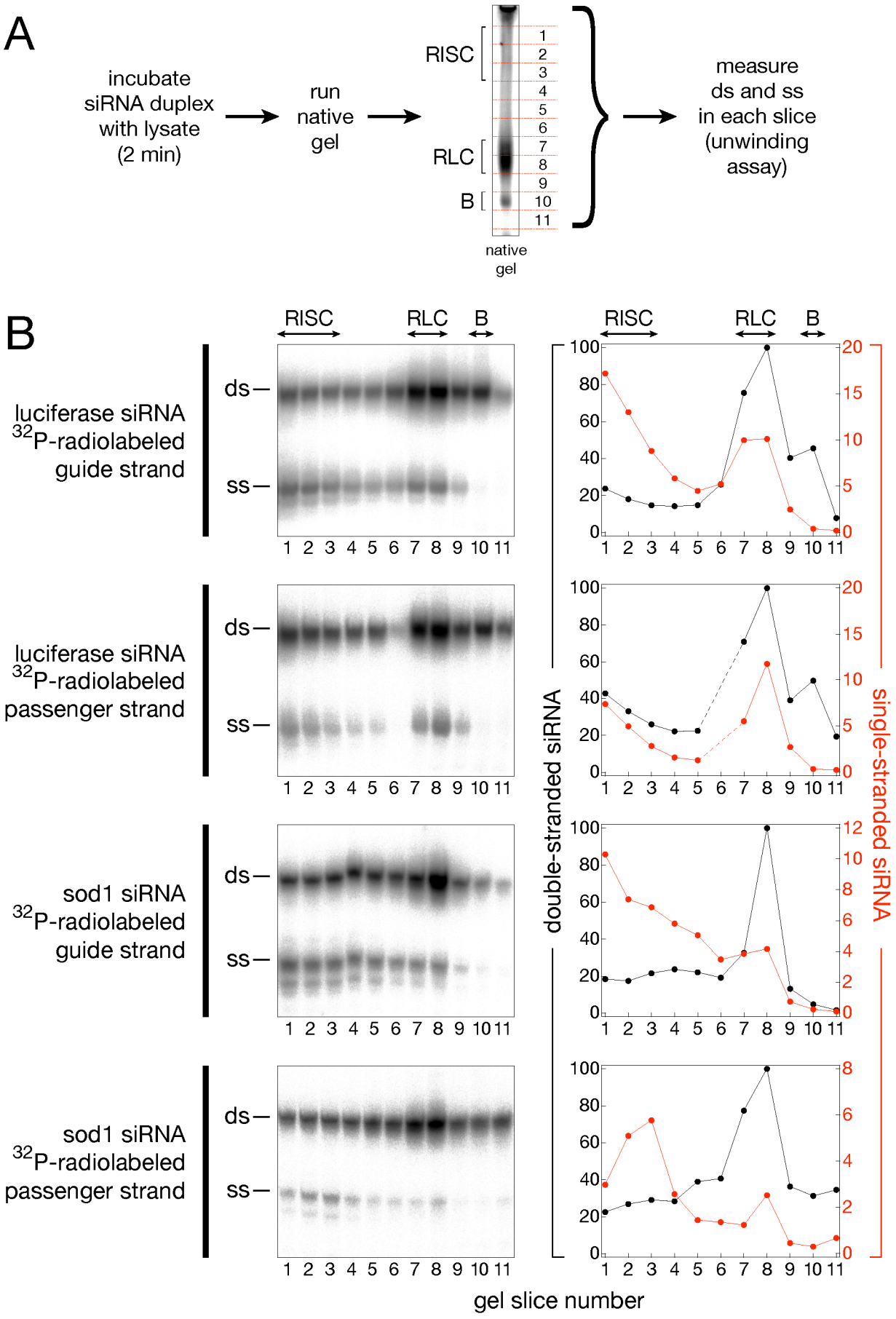
11. Data not shown.

Tomari et al., Figure S1

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11
native gel
measure ds and ss in each slice (unwinding assay)
B
luciferase siRNA 32P-radiolabeled guide strand
luciferase siRNA 32P-radiolabeled passenger strand
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sod1 siRNA 32P-radiolabeled passenger strand
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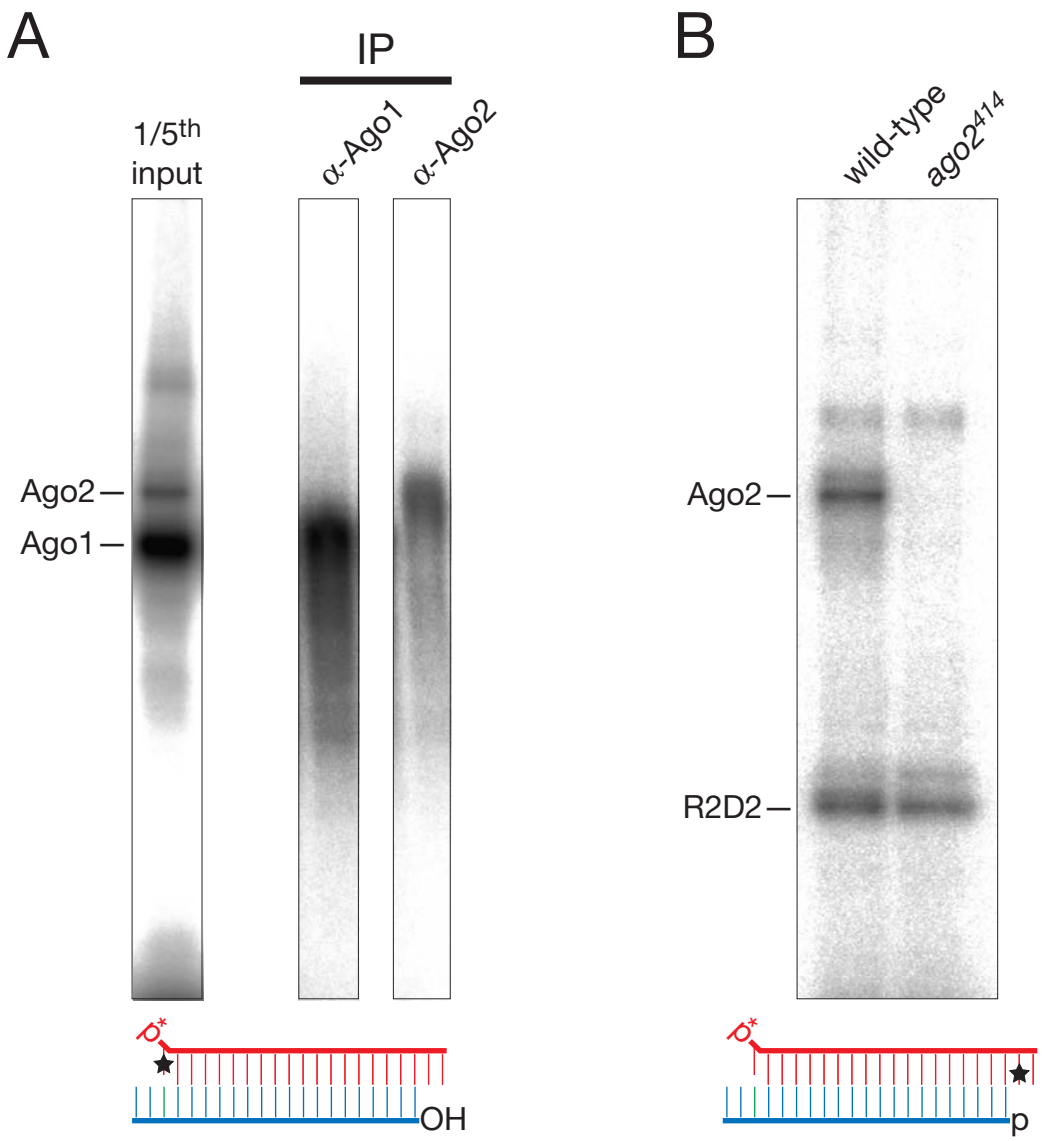
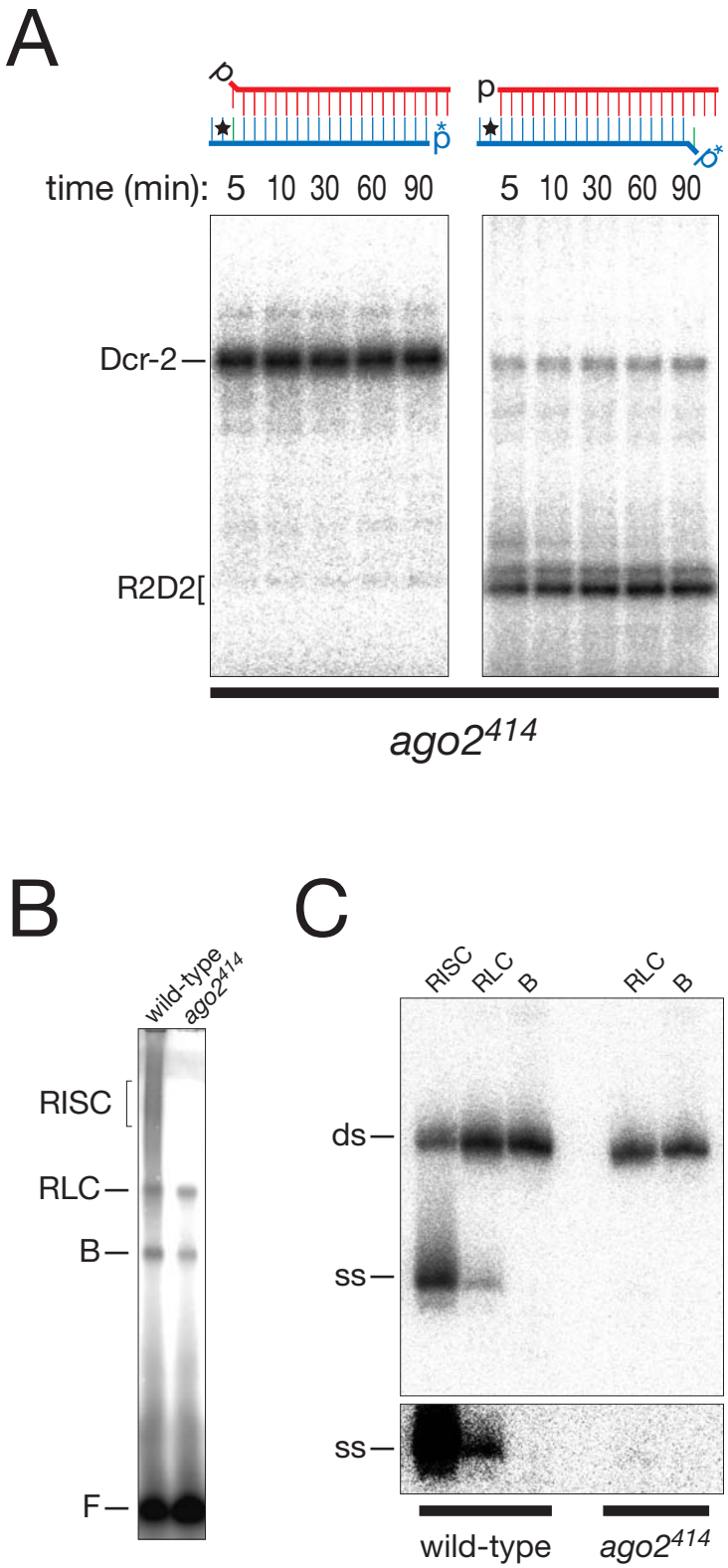
Tomari et al., Figure S3

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Primer

Small silencing RNAs

Christian Matranga and Phillip D. Zamore

Just fourteen years ago, Victor Ambros and coworkers discovered the first microRNA (miRNA). And just eight years ago, small interfering RNAs (siRNAs), the small silencing RNAs that mediate RNA interference (RNAi), were identified and subsequently shown in animals to be derived from a longer double-stranded RNA trigger and to serve as guides for the destruction of complementary mRNAs. The discovery of siRNAs enabled the application of RNAi — itself discovered in plants in 1990 and in animals in 1995 — to mammals, much as the identification in 1998 of double-stranded RNA as the trigger of RNAi enabled the widespread use of RNAi in other animals. Today, tens of thousands of small silencing RNAs have been identified, generating an alphabet soup of small silencing RNA types and sub-types.

Small silencing RNAs have captivated the scientific world — bringing new genetic tools to model organisms, new explanations for regulatory interactions, new methods to pharmaceutical discovery, and new life to the biotechnology industry (where young start-up companies strive to develop siRNA-based drugs). They have even generated unprecedented discussion in the popular press: the cover of the international business magazine, the *Economist,* recently proclaimed small RNAs to be "Biology's Big Bang". For those of us in the RNA silencing field, the rapid progress in understanding the mechanisms and functions of small silencing RNAs and the accelerating discovery of new classes of tiny RNA silencers has been exhilarating.

Despite their functional and biological diversity, all small silencing RNAs function bound to a member of the Argonaute family of proteins. Through their association with Argonaute proteins, small RNAs take on unique properties that allow them to regulate diverse biological processes. Here, we shall consider the three dominant classes of animal small silencing RNAs: miRNAs, siRNAs and PIWI-interacting RNAs (piRNAs; Figure 1).

miRNAs

miRNAs reside in the genomes of plants, animals and viruses, but not, we believe, those of fungi. miRNAs are transcribed as mRNA-like primary (pri-) miRNAs containing ~70 nucleotide long stem–loop structures (Figure 1A). Once excised from the pri-miRNA by the nuclear enzyme Drosha, these stem-loops become precursor (pre-) miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm, where they are cleaved again, by the ribonuclease Dicer, to yield ~22 nucleotide long mature miRNAs containing 5′ phosphate and 3′ hydroxy termini. A small number of pre-miRNAs also double as introns — 'mirtrons' —which are processed first by the pre-mRNA splicing machinery in the nucleus, rather than Drosha, and then by Dicer in the cytoplasm.

miRNAs are an ancient innovation among animals: for example, both of the first two miRNAs discovered, *lin-4* and *let-7,* are conserved from nematodes to humans. The conservation of miRNAs between monocots such as wheat and dicots such as *Arabidopsis* — plants that diverged over two hundred million years ago — suggests an ancient origin for miRNAs in plants, too, and the recent discovery of miRNAs in the green unicellular alga, *Chlamydomonas reinhardtii,* hints that miRNAs could date back to the dawn of photosynthetic eukaryotes. Nonetheless, there is no compelling evidence that plants and animals share any miRNA in common, suggesting that miRNAs arose at least twice in evolution, perhaps from an ancestral RNAi pathway.

Plant miRNAs are nearly perfectly complementary to the mRNAs they regulate, allowing them to direct the Argonaute protein, Ago1, to cleave their targets, which are often members of transcription factor families that regulate leaf or floral development. Both plant miRNAs and siRNAs are 2′-O-methylated at their 3′ termini by the S-adenosyl methionine–dependent methyltransferase HEN1. This modification is thought to protect plant small RNAs from 3′ polyuridylation, a signal likely to promote small RNA degradation. *Chlamydomonas* miRNAs are also methylated, suggesting that the small RNA methylase, HEN1, was present in an ancestral, unicellular plant.

Animal miRNAs are generally not terminally modified, and they are typically less complementary to their target mRNAs than those in plants. Animal miRNAs can direct the Argonaute protein Ago2 to cleave an mRNA target — a process that requires extensive base pairing between the miRNA and its binding site on the mRNA. This form of regulation is rare, with only eight examples identified in mammals among the tens of thousands of predicted miRNA:mRNA regulatory pairs. Instead, most animal miRNAs either block mRNA translation or target mRNAs for destruction by standard mRNA turnover mechanisms.

miRNAs can bind their targets even when they are complementary at just six or seven — very special — contiguous bases. This region of the miRNA, comprising miRNA nucleotides 2 through 7, is known as the 'seed', and is created only when the miRNA is fully engaged with an Argonaute protein. The ability of miRNAs to bind their targets through the seed alone makes target finding difficult, although computational predictions have been surprisingly successful, especially when evolutionary conservation of miRNA-binding sites is used to evaluate preliminary results.

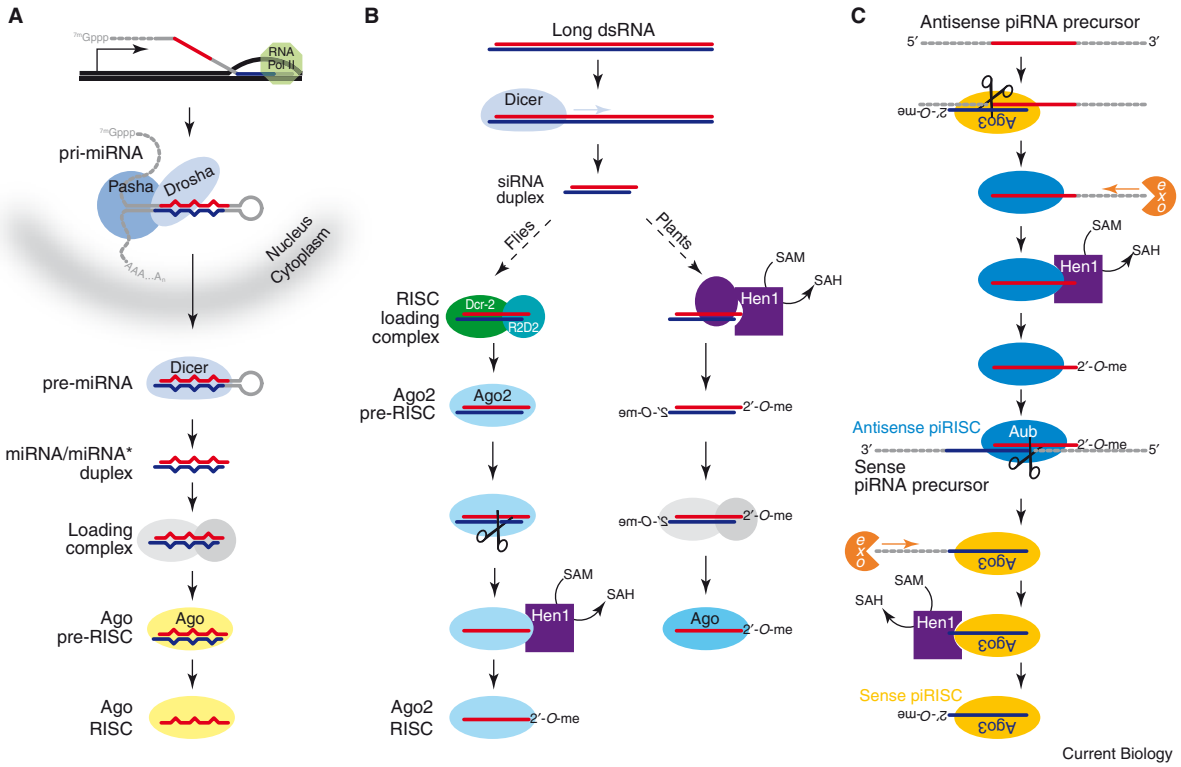


Figure 1. Biogenesis of small silencing RNAs.

(A) In animals, pri-miRNAs are transcribed by RNA polymerase II (or, rarely, by RNA polymerase III), processed in the nucleus into pre-miRNAs by the RNase III enzyme, Drosha, and then exported to the cytoplasm where the pre-miRNAs are converted to miRNA/miRNA* duplexes by a second RNase III enzyme, Dicer. In plants, the nuclear enzyme, DICER-LIKE 1 acts as both Drosha and Dicer. How miRNAs are loaded into Argonaute proteins is not yet known. (B) In the *Drosophila* RNAi pathway, Dicer-2 acts twice: once to make siRNAs from long double-stranded RNA and once to load the siRNA duplex into Argonaute2. (C) A speculative model for the production of piRNAs in animal germ cells.

How miRNAs regulate their mRNA targets remains contentious. Do some miRNAs increase mRNA turnover while others repress translation, or is mRNA instability a consequence of blocking translation for some mRNAs? Which step of translation do miRNAs regulate? Some data suggest that miRNAs block protein translation after the initiation of translation, perhaps by inhibiting polypeptide elongation or even by degrading the nascent peptide. Recent data implicate translational initiation as the regulated step.

In human cells, Ago2 has been proposed to contain a domain that binds the 7-methyl-guanosine cap of mRNA, competing with the binding of eIF4E that is required to recruit ribosomes to a message. Alternatively, miRNAs have been hypothesized to increase the concentration of eIF6 on their target mRNAs, thereby antagonizing ribosome subunit joining, a prerequisite for the assembly of a functional ribosome. Animal miRNA ribonucleoproteins have also been proposed to direct mRNAs to cytoplasmic sites of RNA degradation such as P-bodies or stress granules. In zebrafish, miRNAs promote deadenylation of maternal RNAs at the onset of zygotic transcription, a clever way of clearing unneeded transcripts that might otherwise sequester much needed ribosomes. miRNA-directed deadenylation has also been observed in cultured mammalian cells.

siRNAs

siRNAs guide RNAi, a conserved eukaryotic response to foreign nucleic acids and the primary anti-viral defense for plants and many animals. Since the discovery of siRNAs in 1999 by Hamilton and Baulcombe — as small RNAs, the presence of which correlates with sequence-specific RNA silencing in plants — subclasses of siRNAs — scnRNAs, natsiRNAs, tasiRNAs, casiRNAs, 21Us, tncRNAs — have proliferated. Most are simply siRNAs with unusual functions, while others are not really siRNAs at all.

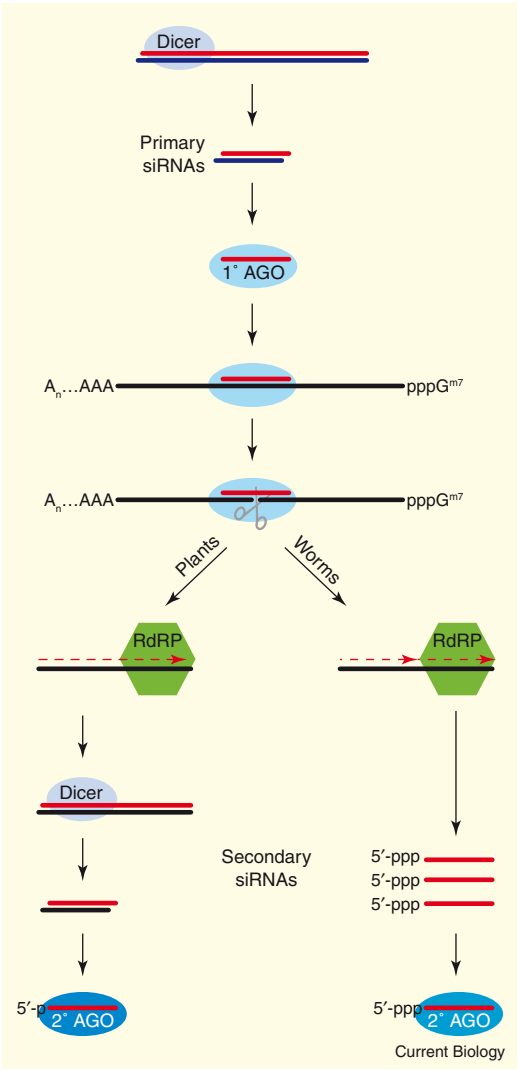
By convention, the names of major classes of small silencing RNAs — miRNAs, siRNAs, and piRNAs — are not intended to encapsulate their regulatory mechanisms or biological functions, but rather to reflect their distinct modes of production. For simplicity, we subdivide siRNAs into just two kinds: those that derive directly from the double-stranded RNA trigger and function without amplification (Figure 1B);, and siRNAs, the function of which requires amplification of an initial long double-stranded trigger RNA by an RNA-dependent RNA polymerase (RdRP; Figure 2).

Canonical, or primary, siRNAs are produced from double-stranded RNA by Dicer, which cleaves processively from an end of the double-stranded RNA, moving in ~21 nucleotide steps. Dicer substrate RNA can arise from viral replication intermediates, convergent transcription, transcripts that self anneal (hairpins), or experimentally introduced double-stranded RNA. The product of dicing is the siRNA, a 21 nucleotide duplex containing 19 base pairs and 2-nucleotide, 3′ overhanging ends. Dicer products contain 5′ phosphate and 2′,3′ hydroxy termini, but may be subsequently modified by S-adenosyl methionine-dependent methyltransferases such as Hen1 in plants and flies to generate a 2′-O-methyl, 3′ hydroxy end. Double-stranded siRNAs are assembled into Argonaute proteins by specific assembly factors that act to orient the siRNA within the Argonaute protein. They generate an effector complex that contains only one of the original two siRNA strands anchored through its 5′ end to a specialized phosphate-binding domain within the Argonaute protein. Such single-stranded, Argonaute-bound siRNAs guide Argonaute proteins to cleave a single phosphodiester bond within the target RNA. This cleavage site is measured from the 5′ end of the siRNA guide, between the target nucleotides paired to siRNA nucleotides 10 and 11.

Plants, fungi, and some animals such as *C. elegans,* encode RdRP enzymes that copy single-stranded RNA into complementary RNA. The RdRPs that function in RNAi generate secondary siRNAs by copying the mRNA targeted by primary siRNAs (Figure 2). Primary siRNAs were originally thought to prime the production of double-stranded RNA by the RdRP copying the target RNA into complementary RNA, which would then be diced into secondary siRNAs. A growing body of evidence argues against this mechanism. Instead, it appears that RdRPs can generate double-stranded RNA by copying a target RNA end-to-end, without the use of primers, but that cleavage of the target RNA by primary siRNAs or miRNAs — probably to remove the cap and poly(A) tail of the target — is a prerequisite for the RdRP to use an RNA as a transcription template. In plants, the new double-stranded RNA is then diced processively to create a phased array of stereotypical siRNAs that regulate other mRNA targets in *trans*.

Figure 2. Amplification of the RNAi response by RNA-dependent RNA polymerases (RdRPs) in plants and worms.

In plants, RdRP enzymes convert target RNAs to double stranded RNA that is cleaved by Dicer into secondary siRNAs. In worms, secondary small RNAs are short transcripts produced directly by RdRPs that use the target of the primary siRNA as a template.



In the nematode *Caenorhabditis elegans,* the RNAi response is initiated by primary siRNA diced from a long, double-stranded RNA trigger. The resulting primary siRNAs trigger the production of secondary siRNAs by an RdRP that uses the target RNA as a template. Worm secondary siRNAs — which are far more abundant than the initial primary siRNAs produced by dicing — are likely produced directly by transcription, without a double-stranded RNA intermediate or dicing. These secondary siRNAs are exclusively antisense to the RNAi target, include sequences both upstream and downstream of the original double-stranded RNA trigger, and begin with the 5′ di- or triphosphate group characteristic of transcription rather than the monophosphate that is the hallmark of dicing. Thus, *C. elegans* secondary siRNAs, unlike those in plants, ought probably be renamed, as the term siRNA was originally intended to describe the small RNA products of Dicer.

Cleavage directed by the primary siRNAs may not be required for secondary siRNA

production in *C. elegans,* as secondary siRNAs can be produced by a single primary siRNA only imperfectly complementary to its mRNA target. Thus, the primary function of worm primary siRNAs may be to recruit an RdRP to the target RNA. Moreover, *C. elegans* secondary siRNAs may be loaded into specialized Argonaute proteins that bind multi-phosphate termini instead of the 5′ monophosphate found on canonical siRNAs.

Plants produce siRNAs from inverted repeat transcripts through the action of two different Dicers. Dicer-like-4 converts double-stranded RNA into canonical siRNAs, 21 nucleotides long, that target mRNAs for cleavage. Dicer-like-3 makes ~24 nucleotide siRNAs, the most abundant class of siRNAs in plants, implicated in directing DNA and histone methylation. All plant siRNA duplexes, both RdRP-dependent and independent, like plant miRNA duplexes, are 2′-O-methylated at their 3′ termini by the HEN1 methyltransferase.

C. elegans also has multiple classes of endogenous siRNAs. 21U-RNAs all begin with uracil and originate from only a few clusters that are specific to chromosome IV. On this chromosome, each 21U-RNA is flanked by a bipartite upstream motif, suggesting that each arises from its own transcript. However, these small RNAs bear 5′ monophosphates, so perhaps their 5′ ends are created by endonucleolytic processing or the initial 5′ triphosphate is post-transcriptionally converted to a monophosphate. The 21U-RNA bipartite motif, but not any of the more than 10,000 individual 21U-RNA sequences, is conserved between *C. elegans* and *C. briggsae.* Thus 21U-RNAs likely act in cis to regulate their own genomic locus. *C. elegans* also produces siRNAs 26 nucleotides long that always begin with guanosine and possess monophosphate 5′ ends. Both the 21U- and 26-mer siRNAs have blocked 3′ termini. The function of 3′ terminal modification is not yet known for any animal small silencing RNA class. Tiny noncoding RNAs (tncRNAs) correspond to the antisense strands of protein coding genes, begin with a 5′ di- or triphosphate, and may be a form of endogenous secondary siRNA produced without dicing by RdRP-catalyzed transcription.

siRNAs nearly always reduce gene expression post-transcriptionally. siRNA function is best understood for flies and mammals, where they guide the Argonaute2 protein to cleave complementary target RNAs at the phosphodiester bond across from siRNA nucleotides 10 and 11, leaving a 5′ fragment with a 3′ hydroxy and a 3′ fragment with 5′ phosphate terminus. Some siRNAs — chiefly those with mismatches engineered in the central region to mimic miRNA:target duplexes — act by controlling an mRNA's translation. siRNAs can also function by activating transcription or translation of mRNA by an unknown mechanism that relies on specific Argonaute proteins.

In the fission yeast, *Schizosaccharomyces pombe,* siRNAs can regulate gene expression both post-transcriptionally and co-transcriptionally. The source of double-stranded RNA from which *S. pombe* siRNAs are diced is thought to be RNA polymerase II transcripts converted to double-stranded RNA by the RdRP, Rdp1, which is a component of a complex that also contains a putative RNA helicase and a nucleotidyl transferase. The resulting siRNAs are thought to recruit an Argonaute-protein complex to nascent transcripts, directing the deposition of repressive chromatin marks, such as histone 3 lysine 9 methylation (H3K9), on centromeric DNA repeats. Similar siRNA-directed nuclear silencing phenomena have also been studied in detail in plants, but their existence and mechanism in animals, especially mammals, is more controversial.

piRNAs

PIWI-interacting RNAs (piRNAs), 24–30 nucleotide long RNAs found in the germ cells of animals, are unique among small silencing RNAs in that they require neither an RdRP nor Dicer for their production (Figure 1C). Instead, they are thought to derive from single-stranded precursor RNAs tens or hundreds of thousands of nucleotides long. piRNAs bind a distinct subclade of Argonaute proteins, the PIWI proteins, which include Piwi, Aubergine, and Ago3 in flies, Smedwi in planaria, Siwi in sea urchins, and Hiwi in humans. *In vitro,* Piwi can cleave a target RNA, suggesting that piRNAs regulate their targets post-transcriptionally, but other evidence suggests they promote heterochromatin assembly in the nucleus. piRNAs were first identified in 2001 in flies, where they repress selfish genetic elements such as retrotransposons.

piRNAs contain 5′ monophosphate and 2′-*O*-methyl, 3′ hydroxy termini. The 2′-*O*-methyl group is added, at least in flies, by the methyltransferase Hen1. In flies, piRNAs are thought to arise from 'master loci' rich in transposons, then act in *trans* to silence dispersed copies of the selfish genetic elements present in the original trigger locus. piRNAs have also been implicated in silencing transposons early in mammalian spermatogenesis. In mammals, however, many piRNAs map to genomic clusters that do not contain repetitive sequences. The functions of these piRNAs are unknown.

piRNAs may be generated by reciprocal cycles of PIWI-protein-catalyzed slicing followed by 3′ trimming by an exonuclease. In *Drosophila,* for example, the first 10 nucleotides of many piRNAs bound to Aubergine, most of which are antisense to transposable element transcripts, can be paired to piRNAs associated with Ago3. (Recall that all Argonaute proteins known to cleave their RNA targets cut after small RNA nucleotide 10.) Nearly all Aubergine-bound piRNAs begin with uracil, whereas Ago3-associated piRNAs, which are almost all in the sense orientation, typically contain an adenosine

at nucleotide 10 — reflecting their base pairing with the first nucleotide of an antisense piRNA. Hence the suggestion that piRNAs are amplified by reciprocal rounds of cleavage, in which Ago3 sense piRNAs direct cleavage of antisense transcripts producing the 5′ monophosphate end of Aub and Piwi antisense piRNAs. A 3′-to-5′ exonuclease could then trim the 3′ end of piRNA transcripts, perhaps acting together with the Hen1 methyltransferase, which might terminate the trimming process by adding a 2′-*O*-methyl group to the 3′ terminus of the mature piRNA.

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Splinted Ligation Incorporating Short Phosphorothioate-Substituted Oligoribonucleotides

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Abstract

Site-specific incorporation of modified ribonucleotides is a powerful tool to dissect the mechanisms of RNA-guided and RNA-catalyzed biological pathways. Here, we show that T4 DNA ligase joins RNA oligomers to RNA as short as a dinucleotide when the ligation substrates are aligned with a DNA splint. Unexpectedly, when we used short ligation substrates containing a single phosphorothioate-modified bond, ligation efficiency was strongly influenced by the position of the thioester relative to the ligated bond. T4 DNA ligase showed a strong preference for the Rp diastereomer. Inhibition of ligation by Sp phosphorothioates is position-dependent; shifting the phosphorothioate linkage allows the equal incorporation of both diastereomers in RNA substrates.

Introduction

T4 DNA ligase joins both DNA and RNA when the substrates are aligned by base pairing to a common DNA strand (Kleppe et al., 1970; Usher et al., 1970; Moore and Sharp, 1993), allowing the site-specific incorporation of modified nucleotides into RNA (Moore and Sharp, 1992). The first use of such splinted-ligation technology ("Moore and Sharp" ligation) was to incorporate phosphorothioates, a modification that introduces a defined chirality into the phosphodiester bond, because a sulfur replaces either the *pro*-Sp or *pro*-Rp non-bridging oxygen (Eckstein, 1970; Moore and Sharp, 1993).

The standard method for introducing a single phosphorothioate diastereomer at a defined position in an RNA begins by using purified dinucleotide containing one diastereomer to prime *in vitro* RNA transcription by a phage polymerase such as T7 RNA polymerase, then using this enzymatically synthesized RNA in a splinted ligation reaction

(Moore and Sharp, 1993). However, advances in the chemical synthesis of RNA now permit the non-enzymatic production of RNAs >100 nts (Shiba et al., 2007). In theory, therefore, it should be possible to use splinted ligation to create RNAs 200 nt or longer in which a single phosphodiester bond is replaced with specific phosphorothioate diastereomer, provided that chirally pure ligation substrates can be prepared.

Here, we report a one-step protocol for the production of such RNAs by using T4 DNA ligase to join three RNA substrates aligned on a synthetic DNA oligonucleotide template. Because the phosphorothioate linkage is contained in a small central RNA as short as two nucleotides, the modified RNA can be readily separated into its diastereomers by high performance liquid chromatography (HPLC), then combined with longer unmodified RNAs in the splinted ligation reaction. Unexpectedly, T4 DNA ligase exhibits stereoselectivity for the Rp diastereomer when phosphorothioate-containing oligoribonucleotides were used, even when the phosphorothioate was not at or adjacent to the ligated bond. T4 DNA ligase used either diastereomer of RNAs 7 nucleotides or longer, provided that the thiophosphate was placed at the fourth phospho-linkage of the 3′ ligation substrate.

RESULTS

Splinted ligation using an RNA dinucleotide substrate containing an internal phosphorothioate

We sought to prepare 21 nt RNA oligos containing single, stereoisomerically pure phosphorothioate substitution, using a one-step splinted ligation method. Each

ribonucleotide was used in a three-way splinted ligation reaction designed to join a ^{32}P-radiolabeled fragment (either 5′or 3′) and an unlabeled fragment (either 3′ or 5′ respectively) to the central modified dinucleotide (Figure 1A). Ligation was inefficient when a dinucleotide containing a racemic mixture of phosphorothioate diastereomers was used as the central (or 5′ substrate) in our initial reactions, yielding primarily a 19 nt deletion product in which the 5′ and 3′ substrates were joined (Figure 1B). When the dinucleotide substrate was in vast excess of the other substrates, the desired 21 nt product was obtained with good yield. Complete T1 nuclease digestion confirmed the identity of both the 19 nt deletion product (Figure 1C) and the 21 nt three-way ligation product that dominated using 50 μM dinucleotide (Figure 1D). Two other side products of the reaction corresponded to ligase-catalyzed 5′ adenylation of the 5′-^{32}P-radiolabeled 3′ ligation substrate (Wang and Silverman, 2006) and a 12 nt ligation products joining the central dinucleotide and the 3′ substrate.

Splinted ligation using RNA substrates containing a single, stereoisomerically-pure phosphorothioate bond

We resolved a racemic mixture of phosphorothioate-substituted ribonucleotide—CpCpsUpA—into its component Rp and Sp diastereomers by reverse-phase HPLC. The identity of the purified oligoribonucleotide diastereomers was confirmed by digestion with ribonuclease P1, which cleaves only the Sp diastereomer, and snake venom phosphodiesterase, which is specific for the Rp diastereomer (Burgers and Eckstein, 1979; Eckstein, 2002). We next performed three-way ligations using the Rp and Sp stereoisomers of this four-nucleotide-long central oligoribonucleotide (Figure 2A).

Surprisingly, the Rp diastereomer was a far better substrate for T4 DNA ligase than the Sp diastereomer (Figure 2B). Addition of Mn^{2+}—a thiophilic cation—did not rescue ligation of the Sp diastereomer (data not shown). Moreover, mismatches between the RNA:DNA hybrid at the nucleotides surrounding the phosphorothioate also did not rescue the ligation defect. A ligation containing the original, racemic 4-mer produced the full-length, three-way ligation product with 50% yield of Rp product, indicating that T4 DNA ligase rejects the Sp dinucleotide and that the Sp diastereomer does not inhibit ligation of the Rp dinucleotide.

We next tested if phosphorothioate stereoselectivity was independent of the length of the modified RNA substrate. We prepared stereoisomerically pure diastereomers for RNA three different oligonucleotides—5, 7, and 10 nts long, each containing a single modification at the n–2 linkage. Ligation efficiency was dramatically lower when the Sp diastereomer was used compared to the all phosphodiester (PO) and Rp controls (Figure 2B). These data suggest that T4 DNA ligase is highly sensitive to modification of the *pro*-Sp group at the n–2 bond.

In contrast to n–2 modified substrates, both diastereomers of a 7 nt RNA bearing a phosphorothioate linkage at the fourth diester linkage, CpCpUpApsCpUpA, were efficiently joined to the adjacent RNA substrates in a three-way splinted-ligation reaction (Figure 3A). Together, our data suggest that substrate recognition or catalysis by T4 DNA ligase is directly influenced by the diastereomeric identity of the diester bond two, but not four, bases from the site of ligation.

To test this idea, we purified the Rp and Sp diastereomers for all of the six possible internal phosphorothioate substitutions for the 7-mer used in Figure 3A. We

examined the potential for each of these twelve RNAs to serve as substrates for two-way splinted ligation by T4 DNA ligase, combining the 7-mer with either the 5′ or 3′ unmodified RNA substrate. When PS-containing 7-mers were used as 5′ substrates, ligation was poor when an Sp phosphorothioate was near the ligated bond—especially at position n–2, but were used more efficiently when the Sp thiophosphate was more distant from the ligation site (Figure 3B). Similar diastereomer-specific positional effects were observed when the phosphorothioate substitutions were made in the longer RNA oligomers and the 7-mer was all phosphodiester, as well as when the RNA substrate sequences were changed; this stereoselectivity is apparent for central RNAs ranging from four to ten (Figure 2B) ribonucleotides in length. Surprisingly, an Rp phosphorothioate had the converse effect: the Rp diastereomer was a less effective 5′ ligation substrate when the substituted diester was more distant from the site of ligation.

When used as a 3′ ligation substrate, all six Rp phosphorothioate-substituted 7-mers were used with efficiencies similar to the control. When the 7-mer served as a 3′ substrate, all the Rp diastereomers ligated efficiently, except for when the thiophosphate was at position +3 or position +6, which ligated with about half the yield of the control. In contrast, the Sp 7-mers were all poor 3′ ligation substrates when the thiophosphate was at any position except for +4 through +6; an Sp phosphorothioate at position +3 was an especially poor substrate (Figure 3C). However, stereoselectivity for 3′ ligation substrates may be sequence-specific: swapping oxygens for thiophosphates in the strand originally all-phosphodiester had no effect on ligation efficiency (Figure 3E). Yet the stereoselectivity for 5′ ligation substrates was not sequence-specific; we see similar positional effects when a thiophosphate was substituted in the oligo that previously

contained an all-phosphodiester backbone (Figure 3D). We conclude that the diastereomer-specific positional effects reflect the stereospecificity of T4 DNA ligase for 5′ ligation substrates.

DISCUSSION

We describe here the use of three-way splinted ligation to produce RNAs containing a single phosphorothioate diastereomer at a specific position. Ligation of all-phosphodiester and Rp-phosphorothioate-containing RNAs proceeded with good yield for RNAs as small as a dinucleotide. In contrast, efficient ligation of Sp-phosphorothioate-substituted RNA was possible only using longer RNAs that allowed the thiophosphate to be placed at non-inhibitory positions.

The requirement to separate the Sp phosphorothioate-substituted diester bond from the ligation site suggests a heretofore unrecognized stereospecificity of T4 DNA ligase. Significant stereospecificity was observed for positions –1 and –2; for the n–2 phosphorothioate-substitution, the Rp diastereomer ligated nearly as well as the all-phosphodiester control, whereas the Sp diastereomer ligated <10% as well (Figures 2 and 3).

In general, phosphorothioate substitutions had a much smaller inhibitory effect when the modified RNA served as the 3′ substrate. An exception to this trend was the strong inhibition observed by an Sp phosphorothioate substitution at position +3 of the 3′ ligation substrate. In the 5′ ligation substrate the Rp diastereomer inhibited ligation more when it was farther from the ligation site, whereas the Sp diastereomer generally inhibited when it was closer to the ligation site. The stronger inhibition by

phosphorothioates 5′ to the ligation site than 3′ substitutions is consistent with previous footprinting studies for T7 DNA ligase suggesting that T7 DNA ligase binds asymmetrically to the ligation site (Doherty and Suh, 2000; Tomkinson et al., 2006). A recent structural analysis of the minimal Chlorella virus DNA ligase shows that nick recognition and substrate binding act through NTPase and OB domain contacts spanning 6 bp 5′ to the nick and 8 bp in the 3′direction (Nair et al. 2007). The OB domain of the Chlorella ligase forms a latch that holds the duplex substrate. The latch makes specific contacts with phosphodiester backbone of the nicked substrate, engaging a 2-nt segment of the 3′ OH-bearing 5 fragment through hydrogen bonds to the vicinal phosphates (Nair et al. 2007). This may explain why sulphur substitution—specifically the Sp diastereomer—at these two diesters inhibits ligation by the related T4 DNA ligase.

We anticipate that the method described here will find broad utility in constructing site-specifically modified RNAs from three or more chemically synthesized RNA fragments. We envision that many diverse modified bases, backbones, and sugar substitutions can be incorporated into longer RNA molecules by the use of stereochemically-defined dinucleotide ligation substrates.

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Materials and Methods

General Methods and oligonucleotides

RNA (Dharmacon) and DNA (IDT DNA) oligos were prepared by standard synthesis.

RNAs were labeled using polynucleotide kinase (New England Biolabs) and γ-^{32}P-ATP (NEN). Sequences of synthetic RNA and DNA oligonucleotides are available in Table S1.

Reverse-phase HPLC purification of phosphorothioate diastereomers

RNA oligonucleotide containing single phosphorothioate (PS) modification were ethanol precipitated, resuspended in 0.1M NH_4OAc (Buffer A), and 50 μg of RNA injected onto a Targa C18 HPLC column (Nest Group). The column was eluted with a 0-15% gradient of Buffer B (0.1 M NH_4OAc, 50% [v/v] acetonitrile) over 15 min followed by a 15-35% of Buffer B gradient over 45 min. Purified diastereomer peak fractions were pooled, lyophilized, then redissolved in water and lyophilized again, for three cycles of lyophilization, flashfreezing in liquid nitrogen after each time dissolution in water. Sample recovery was about 50%. Diastereomer identity was established by nuclease P1 treatment. Samples were treated with 2 units nuclease P1 (US Biological) for 60 min at 37°C, dephosphorylated with calf intestinal phosphatase (New England Biolabs), and then the resulting nucleosides were separated on a Targa C18 HPLC column (Nest Group), run first in 0-8% Buffer B over 25 min, followed by elution with a 8-30% Buffer B over 35 min. The characteristic nuclease P1-resistant Rp dinucleotide eluted from the column at predicted time. To confirm the identity of the dinucleotide, chiral dinucleotide standards were prepared by separating a racemic mixture of a PS-modified dinucleotide (Dharmacon) by reverse phase-HPLC.

Splinted Ligation of RNA using T4 DNA Ligase

RNA was ligated using complementary DNA oligonucleotides containing 5 nt-3′overhangs. A typical reaction contained 20 units RNAsin Plus (Promega), 15 units T4 DNA ligase (Fermentas), and a final concentration of each of the following: 1X T4 DNA Ligase buffer, 0.3μM DNA splint, and 0.4μM RNA substrates. When using RNA oligos ≤ 4nts in length, we increased the concentration 2-100 fold versus other RNA substrates in the reaction. Reactions were incubated at 16˚ C overnight, quenched by adding 2X Proteinase K buffer (200 mM Tris-Cl [pH 7.5], 25 mM EDTA, 300 mM NaCl, 2% [w/v] SDS), 2 mg/ml Proteinase K, and 1 μg glycogen, incubated for 15 min at 65°C, extracted with an equal volume of phenol/chloroform (1:1), and the RNA precipitated with 3 volumes absolute ethanol. RNAs were resolved by electrophoresis through a 15% denaturing (19:1) polyacrylamide gel, the gel dried under vacuum, and detected with a FLA 5000 phosphorimager (Fuji).

RNase T1 digestion of ligation substrates

Ligation products (Figure 1B) were resolved through a 15% denaturing urea polyacrylamide gel, the 19 nt—no dinucleotide reaction—and 21 nt—excess dinucleotide—products were excised, and eluted overnight in 2x Proteinase K buffer. The samples were then extracted with phenol:chloroform (1:1), precipitated with 3 volumes of ethanol, the precipitate collected by centrifugation, washed with 80% (v/v) ethanol, and the pellets dissolved in 50 mM Tris-HCl (pH 7.5), 1mM EDTA. RNA samples were incubated with 100 units of RNAse T1 (Ambion) for 30 min at 37˚ C. T1 reactions were stopped with an equal volume of 98% formamide buffer.

Figure Legends

Figure 1. Incorporation of a RNA dinucleotide in a three-way splinted ligation. (A) A 31 nt-long DNA splint (blue) and 8 and 11 nt long RNA oligos (red) were used as substrates to ligate to a central phosphorothioate-modified RNA dinucleotide. (B) Ligation of full length 21 nt products required excess central dinucleotide. (C) 19 nt (no dinucleotide) and 21 nt (50μM dinucleotide) ligation products were excised from the gel and subjected to RNAse T1 digestion.

Figure 2. T4 DNA ligase exhibits stereoselectivity for RNA substrates containing Rp phosphorothioate diastereomers at position n-2. Each of diastereomer of a 4, 5, 7 and 10 nt oligo was tested for ligation efficiency when used as a central RNA between 5′ (UAUACAA) and 3′ (*pCUAUUCUCUU) RNA substrates and complementary DNA splint bearing 5 nt-overhangs. Total fraction central RNA ligated to 3′ RNA substrate was measured for each to determine stereoselectivity. PO, phosphodiester; Rp, Rp phosphorothioate diastereomer; Sp, Sp phosphorothioate diastereomer.

Figure 3. Testing T4 DNA ligase stereoselectivity at each diester of 7 nt long RNA substrate in two-way ligations. (A) Multi-way ligation using diastereomers of central 7mer RNA containing a single phosphorothioate at the 4th diester linkage. Two-way—central plus 3′RNA—and three-way ligation were equally efficient for each of the three central RNA substrates used. Two way splinted ligation using RNA substrates containing a single, stereomerically-pure phosphorothioate in either the 5′ (B) or 3′ (C)

RNA fragment. Fraction ligation was normalized to the all-phosphodiester control. (D, E) Preference for particular RNA sequence was tested by substituting phosphorothiotes in the original phosphodiester oligo for both the 5′ and 3′ RNA substrates used in B, C.

Table S1. List of DNA and RNA oligo sequences

DNA:

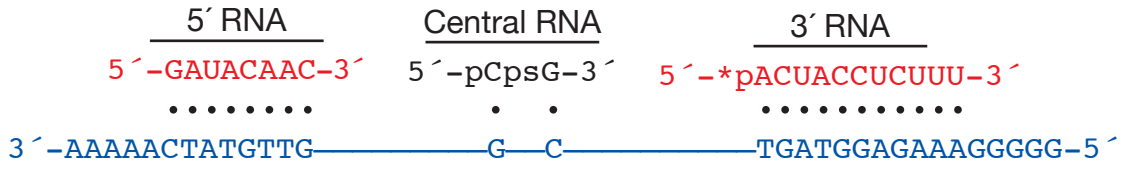
Sequence:	Name	Figure
5´-GGGGGAAAGAGGTAGTCGGTTGTATCAAAAA-3´	31.443m	1
5´-GGGGGAAGGAGGTAGTAGGTTGTATAAAAAA-3´	31.443	2
5´-GGGGGAAGGAGGTAGTAGGTTTGTATAAAAAA-3´	32.443c	2
5´-GGGGGAAGGAGGTAGTAGGTTGTTGTATAAAAAA-3´	34.443c	2
5´-GGGGGAAGGAGGTAGTAGGTTGTATTTGTATAAAAAA-3´	37.443	2
5´-GGGGGCGAATATATAGTAGGTTGTATAAAAAA-3´	32.443	3

RNA:

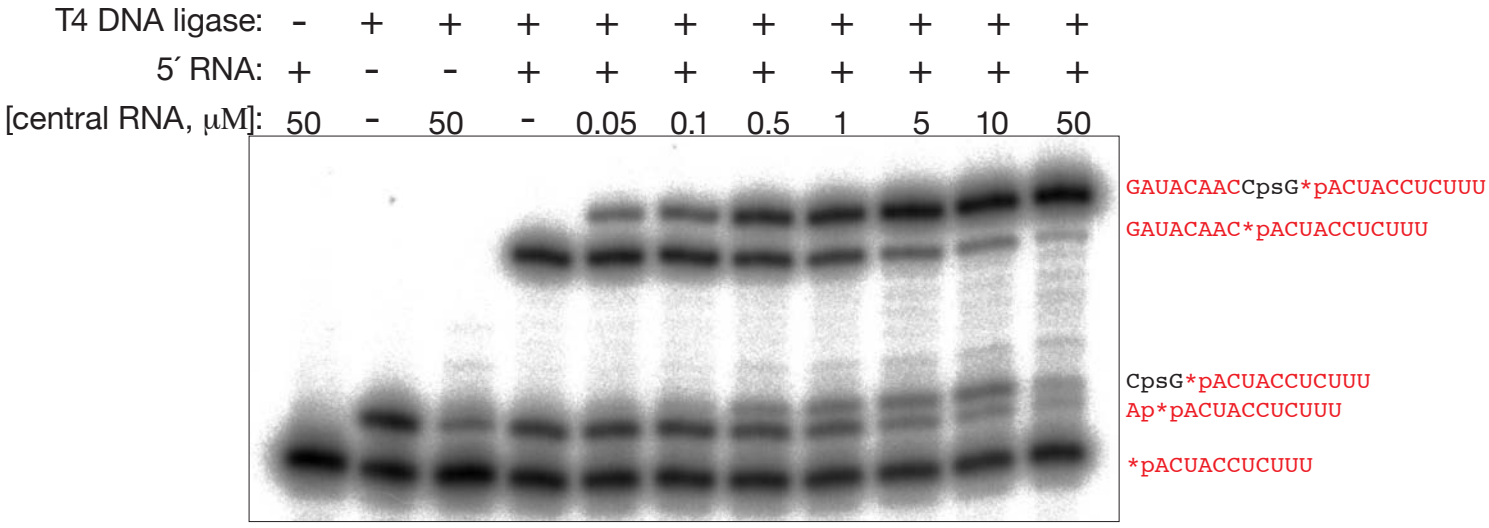
Sequence:	Name	Figure
5´-C*G-3´	2.443	1
5´-CC*UA-3´	4.443	2
5´-ACC*UA-3´	5.443	2
5´-CAACC*UA-3´	7.443mm	2
5´-AUACAACC*UA-3´	10.443	2
5´-GAUACAAC-3´	8.443m	1
5´-ACUACCUCUUU-3´	11.443m	1
5´-UAUACAA-3´	7.443 (5′)	1, 2, 3
5´-CUACCUCCUU-3´	10.443 (3′)	1, 2, 3
5´-CCUACUA-3´	7.445 (mid)	3
5´-UAUAUUC-3´	7.12	3
5´-UAUAUUCG-3´	8.12	3

*=phosphorothioate substitution

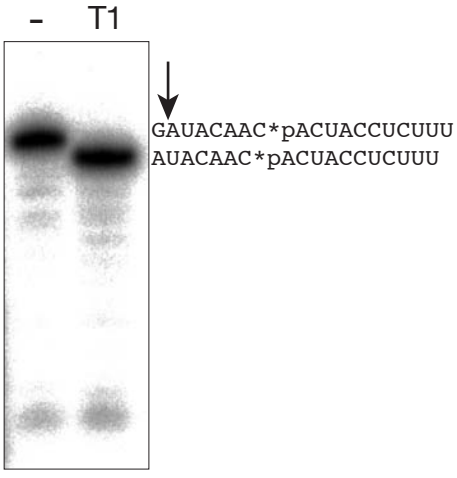
A


5´ RNA
Central RNA
3´ RNA
5´-GAUACAAC-3´
5´-pCpsG-3´
5´-*pACUACCUCUUU-3´
3´-AAAAACTATGTTG
G
C
TGATGGAGAAAGGGGG-5´

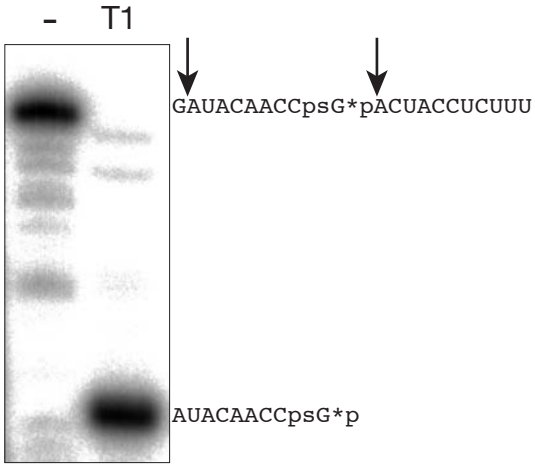
B

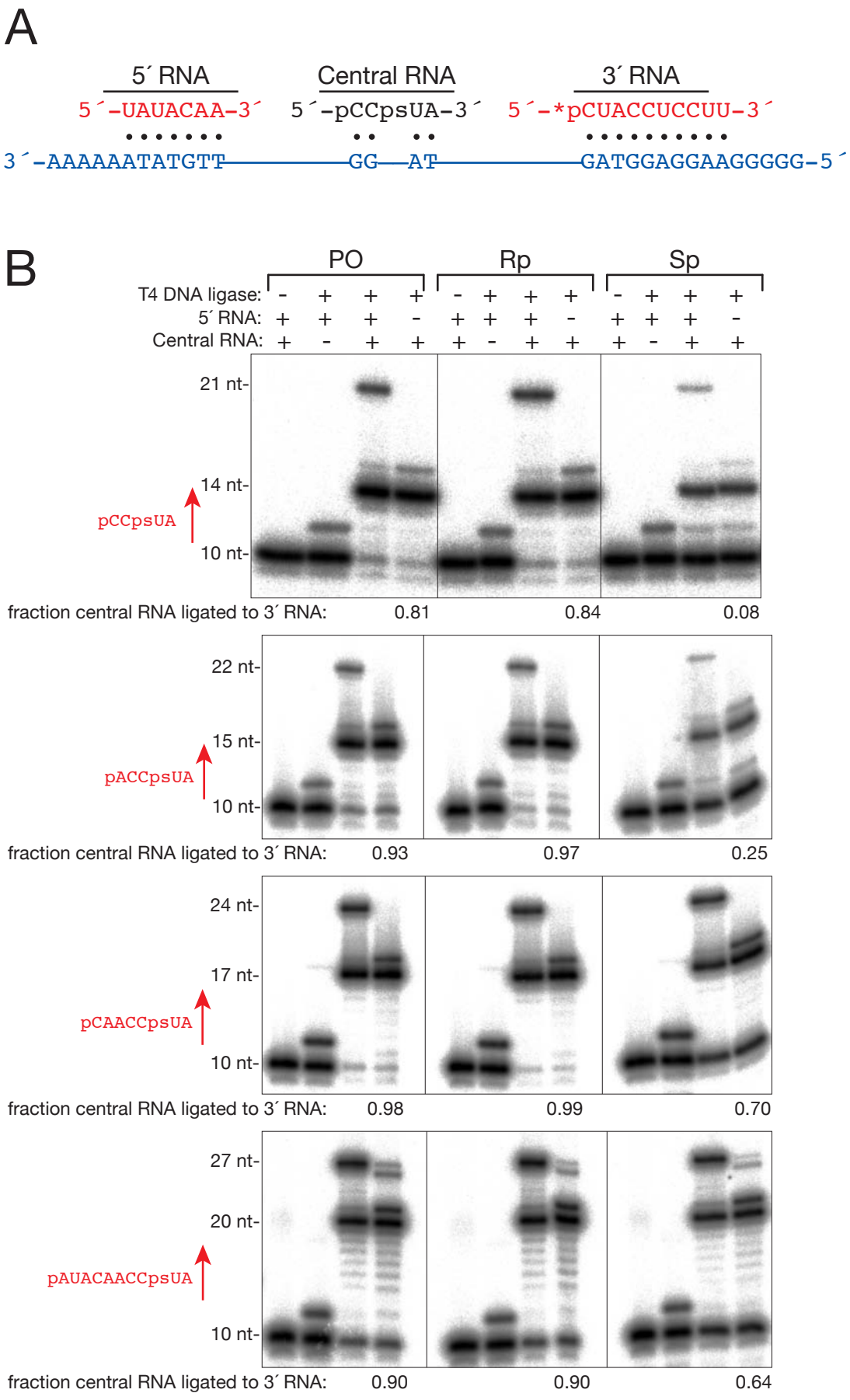

T4 DNA ligase: - + + + + + + + + + +
5´ RNA: + - - + + + + + + + +
[central RNA, μM]: 50 - 50 - 0.05 0.1 0.5 1 5 10 50
GAUACAACCpsG*pACUACCUCUUU
GAUACAAC*pACUACCUCUUU
CpsG*pACUACCUCUUU
Ap*pACUACCUCUUU
*pACUACCUCUUU

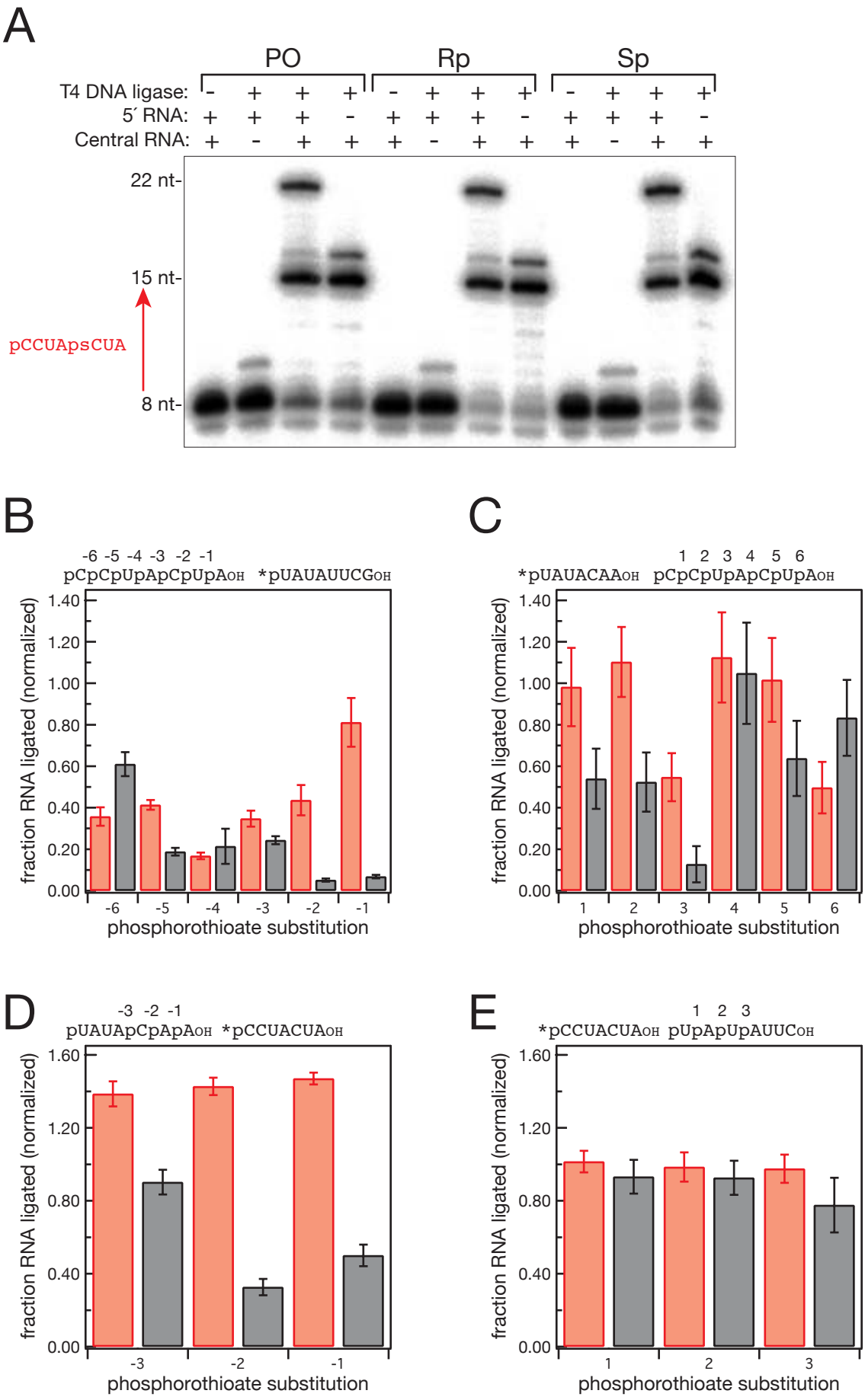
C


- T1
GAUACAAC*pACUACCUCUUU
AUACAAC*pACUACCUCUUU

D


- T1
GAUACAACCpsG*pACUACCUCUUU
AUACAACCpsG*p


A
5´ RNA
Central RNA
3´ RNA
5´-UAUACAA-3´
5´-pCCpsUA-3´
5´-*pCUACCUCCUU-3´
3´-AAAAAATATGTT
GG AT
GATGGAGGAAGGGGG-5´
B
PO
Rp
Sp
T4 DNA ligase:
5´ RNA:
Central RNA:
21 nt-
14 nt-
pCCpsUA
10 nt-
fraction central RNA ligated to 3´ RNA: 0.81 0.84 0.08
22 nt-
15 nt-
pACCpsUA
10 nt-
fraction central RNA ligated to 3´ RNA: 0.93 0.97 0.25
24 nt-
17 nt-
pCAACCpsUA
10 nt-
fraction central RNA ligated to 3´ RNA: 0.98 0.99 0.70
27 nt-
20 nt-
pAUACAACCpsUA
10 nt-
fraction central RNA ligated to 3´ RNA: 0.90 0.90 0.64


A
PO
Rp
Sp
T4 DNA ligase:
5´ RNA:
Central RNA:
22 nt-
15 nt-
8 nt-
pCCUApsCUA
B
-6 -5 -4 -3 -2 -1
pCpCpUpApCpUpAOH *pUAUAUUCGOH
fraction RNA ligated (normalized)
phosphorothioate substitution
C
1 2 3 4 5 6
*pUAUACAAOH pCpCpUpApCpUpAOH
fraction RNA ligated (normalized)
phosphorothioate substitution
D
-3 -2 -1
pUAUApCpApAOH *pCCUACUAOH
fraction RNA ligated (normalized)
phosphorothioate substitution
E
1 2 3
*pCCUACUAOH pUpApUpAUUCOH
fraction RNA ligated (normalized)
phosphorothioate substitution